AN IN VITRO COMPARISON OF THE OSTEOGENIC POTENTIAL OF EQUINE STEM CELL POPULATIONS AND SUBPOPULATIONS FROM MULTIPLE TISSUE SOURCES

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ABSTRACT

An *in vitro* comparison of the osteogenic potential of equine stem cell populations and subpopulations from multiple tissue sources was made to identify the ideal equine donor tissue as a source of MSCs to promote bone healing. Equine muscle tissue– and periosteal tissue–derived cells where characterized as mesenchymal stem cells (MSCs) and their proliferation capacity and osteogenic potential was assessed in comparison with bone marrow– and adipose tissue–derived MSCs. Cells were isolated from skeletal muscle, periosteal, and adipose tissues, and sternal bone marrow aspirates. Morphology, adherence to plastic, trilineage differentiation, and detection of stem cell surface markers CD44 and CD90 were used to characterize cells as MSCs. Osteogenic potential of MSCs was measured by osteocalcin gene expression. Mesenchymal stromal cell cultures were counted at 24, 48, 72, and 96 hours to determine tissue-specific MSC proliferative capacity. Muscle MSCs (MMSCs), periosteum MSCs (PMSCs), and adipose MSCs (AMSCs) proliferated significantly faster than did bone marrow MSCs (BMSCs) at 72 and 96 hours.

Non-equilibrium gravitational field-flow fractionation (GrFFF) was validated as a method for sorting MSCs from four donor sources (muscle, periosteum, bone marrow, and adipose tissue) into subpopulations. Aliquots of MSCs from each tissue source were consistently separated into 6 fractions by continuous flow (GrFFF proprietary system) and these fractions remained viable for use in further assays. Absorbencies (OD) were compared, and trilineage assays performed. Statistical analysis of the fraction absorbencies (OD) revealed a P-value of <0.05 when fraction 2 and 3 were compared to fractions 1, 4, 5, and 6.

GrFFF was used to sort MMSCs and BMSCs into subpopulations and perform assays allowing comparison of their osteogenic capabilities. Aliquots of MMSCs and BMSCs were sorted into 5 fractions using non-equilibrium GrFFF. Pooled fractions were cultured and expanded for assays including: flow cytometry, histochemistry, bone nodule assays, and real time PCR to identify upregulation of osteocalcin, RUNX2, and osterix. There was significant upregulation of osteocalcin, RUNX2, and osterix for the BMSC fraction 4 with $P<0.00001$ indicating high osteogenic potential. Flow cytometry revealed different cell size and granularity for BMSC fraction 4 and MMSC fraction 2 when compared with unsorted controls and other fractions. Histochemistry and bone nodule assays revealed positive staining nodules but no significant differences between tissues or fractions.

It was concluded that 1) equine muscle and periosteum are sources of MSCs that have osteogenic potential comparable to that of equine adipose- and bone marrow–derived MSCs, 2) non-equilibrium GrFFF is a valid method for sorting equine MMSCs, PMSCs, BMSCs, AMSCs into subpopulations that remain viable and 3)subpopulations of MSCs exist and have different osteogenic capacities within equine muscle and bone marrow derived sources. These findings are important contributions to equine stem cell therapy and bone healing in veterinary medicine.

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I dedicate this PhD to my loving and supportive husband, Bradley Back, and wonderful 3 year old son, Tyler Joseph Back.

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LIST OF ABBREVIATIONS

AMSC	Adipose derived MSC
BMSC	Bone marrow derived MSC
BSA	Bovine serum albumin
FACS	Fluorescence-activated cell sorting
FFF	Field flow fractionation
FITC	Fluorescein isothiocyanate
GrFFF	Gravitational field flow fractionation
MEM	Minimal essential medium
MMSC	Muscle derived MSC
MSC	Mesenchymal stem cell
PMSC	Periosteal derived MSC
qPCR	Real time quantitative polymerase chain

1 GENERAL INTRODUCTION

1.1 The problem

Rigid internal fixation and external coaptation are adequate techniques to allow natural healing of most fractures in most species.(1-4) When faced with a bone defect that exceeds the natural healing capabilities of the animal, such as a delayed or a non-union, many strategies have been implemented to promote healing.(5) Bone grafts, and bone marrow injections are primarily performed in veterinary species while *in vitro* expansion of autologous bone marrow derived mesenchymal stem cells (MSCs) and later injection has been successful in recent human cases of non-union.(6) Many new approaches are based on bone producing MSCs, and cell based therapies, providing surgeons with more treatment options in the near future.(7)

When MSCs are injected they tend to migrate through the body by means of chemotaxis or 'homing' to tissues that are need of repair. When intended to stay at the site of injection, scaffolds are used to keep MSCs in place and give them a structure in which to congregate.(8) The ideal scaffold is biocompatible, absorbable, osteoconductive, osteoinductive, highly porous, and reproducible.(9) Some of the scaffolds currently under investigation are hydroxapatite,(10) tricalcium phosphate,(11) demineralized bone,(12-14) coral,(15) collagen,(16) and hydrogel.(9) Scaffolds can also serve as vehicles to deliver various factors to enhance bone healing.(10) Bone metabolism and homeostasis are regulated by many hormones and growth factors such as parathyroid hormone (PTH), insulin-like growth factors (IGFs), platelet-derived growth factor (PDGF), fibroblast growth factors (FGFs), vascular-endothelial growth

factors (VEGFs), transforming growth factors (TGF), and bone morphogenetic proteins (BMPs).(17) Knowledge of the physiological roles of these components of bone formation makes them useful targets for optimization of osteogenesis. However, the gold standard MSC scaffold/growth factor combination in osteogenesis has not been determined but is likely to be major factor in successful promotion of bone healing.(9)

The challenge of timely bone healing is of particular significance in the equine industry. Catastrophic breakdown is a major source of wastage in racehorses, with as many as 67%-89% of deaths attributed to exercise-related injury.(18-20) Fractures account for 71% in UK, 88% in California, and 92.7% in Mid-west USA, of fatal musculoskeletal injuries in racing Thoroughbreds.(18, 19, 21, 22) The initial surgical stabilization of equine fractures with metal plates and screws is costly at $2,000-$10,000(23) and cost increases with the onset of complications the accompanying prolonged hospital stay.(24) Due to their size and temperament, horses must remain weight bearing on all four limbs during the healing process.(25) If not comfortable on all four limbs horses are prone to the development of contralateral limb laminitis due the uneven weight sharing between limbs while recovering.(26) Many horses tolerate slings, which lessen the load on both the fractured and contralateral limbs, but patient selection is key and therefore they are not always an option.(27) This leaves the surgical repair susceptible to breakdown and failure because the constant loading of implants often causes fatigue and ultimately failure of the metal screws and plates.(28, 29) The constant loading combined with the fact that equine fractures heal much more slowly than those of other veterinary species,(30) lead to life threatening complications and a poor prognosis for most equine fracture patients. In addition, many adult equine

fractures are high energy fractures with much soft tissue trauma.(31) Without viable soft tissues covering a fracture repair, bone repair has been shown to be delayed.(32) Other postoperative complications include those attributable to inadequate tissue coverage and blood supply, as well as bacterial contamination: incisional infection, implant infection, and non-union of fracture(11); those due to uneven weight sharing and cyclic loading: contralateral limb laminitis, and implant failure or loosening/ breakage; and those due to stress and antimicrobial therapy; colic, and diarrhea.(33, 34)

If equine fracture healing rates could be increased, the prolonged hospital stays could be shortened and complications such as non-union of fractures and contralateral limb laminitis would decrease, thus increasing survival rates and making the initial fee of fixation a more worthwhile investment to owners. To this end, veterinary researchers are active in areas of research for infection prevention, improving implant design, and methods of increasing osteogenesis itself. (11, 27, 35, 36) Conventionally, bone healing is best enhanced with the current gold standard, cancellous bone grafts.(35) However, the successful utilization of a cancellous bone graft is limited by supply, the additional surgical site required, increased time required for harvesting and placement, cost, and potential donor site morbidity associated with its harvest.(5) Therefore, it would be beneficial to our veterinary patients to identify other potential sources of graft substitutes such as mesenchymal stem cells (MSCs) that could enhance osteogenesis.

1.2 MSC research to date – sources and applications

Stem cell research aimed towards tissue regeneration is an expanding field in both human and veterinary medicine.(37) MSCs are defined by their capacity to differentiate into a variety of other cell types including fat, cartilage, bone, muscle, and nerve cells.(38) There is evidence in the lab animal,(39-42) and human literature to support the notion that MSCs may be used to enhance healing and repair of musculoskeletal defects including those affecting bone.(6, 43, 44) Human bone marrow derived MSCs have been shown to promote bone healing in long bone and skull defects in laboratory animal models.(45, 46) In large animals, sheep bone marrow derived MSCs implanted in porous hydroxyapatite ceramic carriers also showed greater bone deposition in a shorter time period, in a tibial ostectomy model, when compared to the carrier alone.(47) In horses, equine MSCs have been used *in vivo* to improve cartilage healing,(48) and tendon healing,(49, 50) but bone healing still requires an *in vivo* trial to assess efficacy. The osteogenic potential of bone marrow derived MSCs has been the most thoroughly researched donor source.(51) Details about bone marrow derived MSCs and osteogenesis, such as proper culture conditions,(52, 53) scaffold effects,(54) differentiation potential, (51) and growth characteristics,(55) have been elucidated. New insight is necessary into tissue sources that are easily harvested and yield high numbers of MSCs. Therefore, new research into the osteogenic potential of other potential graft substitutes, such as other sources of MSCs, are a valid and necessary next step in the search for methods to increase the rate of equine bone healing.

Research in lab animals and humans has revealed many potential donor sources of MSCs; bone marrow and adipose tissue,(56) derivations of umbilical cord,(57-59)

periosteum,(60) synovial membrane,(61) muscle,(62) dental pulp,(63) and cardiac muscle.(64) In horses the most commonly used sources are bone marrow and adipose tissue,(65) but less conventional sources have been explored including; umbilical cord blood and tissue,(66-68) Wharton's Jelly,(69) amnion,(70) amniotic fluid,(71) tendon and muscle,(72) and blood.(73) Thus, a major focus in the following studies was to assess the osteogenic capacities and to characterize periosteum and muscle as novel donor sources of MSCs in horses.

1.3 Novel sources of MSCs

In vitro studies identified periosteum,(74) and muscle tissues (75) as potential sources of equine osteogenic cells for use in cell based therapy. Results have shown that MSCs can be isolated and expanded from periosteum and muscle tissues. The MSCs from periosteum and skeletal muscle also show osteogenic differentiation when cultured in conventional osteogenic medium. Because properties of the donor tissues should include ease of harvest, minimal morbidity, optimum proliferation capacity and osteoblastic activity, both periosteum and skeletal muscle show promise for use in cell-based therapy similar to that of bone marrow and adipose tissue which have been investigated more thoroughly.(55) Many groups have interest in the comparison of tissue sources for MSC harvest and their subsequent osteogenic capacity, but none have identified a tissue source of MSCs that are clear leaders in osteogenesis.(51, 76) Identification of the optimal source of MSCs with the best osteogenic potential may prove critical in moving

the basic science research toward successful clinical cell based therapy to promote bone healing.

1.4 Stem cells

It is generally accepted that stem cells can be best defined by two requirements: they must sustain their own population, and be capable of either toti-, pluri-, or multipotency.(9) Totipotency is the ability of a single cell to differentiate into all of the cells in an organism. Pluripotency is the ability of a cell to differentiate into cells of any of the three germ layers; endoderm (stomach lining, gastrointestinal tract, and lungs), ectoderm (skin and nerves), and mesoderm (muscle, bone, blood). Multipotency is the term applied to cells that have the ability to differentiate into multiple but limited different cell types (cartlage, fat, and bone). That being said, the terminology surrounding stem cell therapy can be misleading as there is a lack of standard terms used throughout publications.(9) This makes comparing studies a challenge. When strictly defined, multipotent cells that are derived from adult tissues are best described as mesenchymal stromal cells, not mesenchymal stem cells.(9) This distinction is made to avoid use of the word ‘stem’ when the cell is multipotent, not pluripotent, and cannot self renew indefinitely.(77) However, in an effort to be consistent with the majority of the published literature that refers to multipotent cells that are derived from adult tissues as mesenchymal stem cells, the studies in this thesis follow suit.

MSCs also display a bimodal effect on the immune system by secretion of bioactive factors that are trophic and immunomodulatory.(78) Trophic activities of MSCs help to stimulate angiogenesis, thus reducing apoptosis, recruit host progenitor

cells, and minimize fibrosis.(79) The immunomodulatory effects prevent lymphocyte awareness of the wounded tissue by interacting with its lymphocyte B cells, T cells, natural killer cells, dendritic cells, macrophages/monocytes, and neutrophils, thus preventing autoimmunity.(78) This down regulation of the immune response allows for the application of allogeneic MSCs without rejection in a variety of clinical situations.(80, 81) This broad range of trophic and immunomodulatory capabilities make MSCs highly desirable for uses in medical applications.

1.4.1 Embryonic stem cells vs. mesenchymal stem cells

The greatest potential for differentiation exists in the youngest stages of embryologic development of the cell mass.(37) Embryonic stem cells (ESCs) are totipotent through the 8 cell stage of the morula;(82) therefore, at that time each cell has the ability to become any cell of the fetus or the fetal membranes. At the subsequent blastocyst stage, cells of the inner cell mass are pluripotent, having the potential to differentiate into any cell in the three germ layers.(83) After birth, stem cells are classified as adult instead of embryonic, and as multipotent instead of pluripotent, having a limited potential to differentiate into multiple tissue lineages including: chondrogenic, adipogenic, and osteogenic.(83)

While related, there are several other differences between ESCs and MSCs. *In vitro* culture techniques are different for ESCs and MSCs. ESCs are non-adherent on plastic culture dishes and therefore must be grown on a layer of primary feeder cells.(84) MSCs are adherent on plastic culture dishes and require no feeder cell layer.(85) ESCs

have the ability to be cultured for extended periods of time while MSCs have a limited lifespan.(85) This lifespan varies with tissue source of MSCs.(38) MSC and ESC based therapies are promising to be some of the most valuable of the new technologies available for bone healing.(86)

1.4.2 Induced pluripotent cells

Induced pluripotent stem (iPS) cells are adult mesenchymal stem cells that have been reprogrammed to be pluripotent using viral vectors that induce the expression of oncogenes in the candidate cell.(87) iPS cells are functionally similar to ECSs in many ways. iPS cells have been found to be identical to ESCs in morphology, proliferation, gene expression, in vitro differentiation and teratoma formation.(87) Both have the potential to replicate indefinitely without differentiation and are capable of becoming any tissue in the body, but iPS cells are created from post-natal cells and circumvent the controversial dilemma faced by ESCs as they are harvested from adults not embryos.(88) This technology has created iPS cells from many species; murine,(89, 90) porcine,(91) ovine,(92) equine,(93-95) human (96) and many tissue sources; stomach, liver, skin,(97) prostate,(98) dental tissue,(99) urinary tract cells.(100) The potential for use of these cell lines is powerful as they present a way to create patient- and disease-specific stem cells from an adult source.(87) Several studies are examining the safety of iPS cells,(101) and others have found their immunogenicity to be nonexistent.(88, 102) As a result, they are presently a very important area of research.(88)

1.4.3 Osteoblastic stem cells

The osteoblastic stem cell lineage is of great interest to researchers who seek to hasten bone healing for it is the cell lineage responsible for the production of bone.(84, 103) Understanding the mechanisms of bone production by these cells is crucial. The stages of differentiation begin with the MSC which first matures to an osteoprogenitor cell that is committed to the osteogenic lineage, and is still self renewing. Next, osteoprogenitors become preosteoblasts. Preosteoblasts only retain a limited capability to proliferate and start to stain positive for alkaline phosphatase enzymatic activity, indicating that this is a reliable early marker of the osteoblastic lineage.(104) Developmentally, preosteoblasts mature into osteoblasts which can be distinguished by their cuboidal shape, lack of ability to divide, and their expression of bone matrix proteins such as bone sialoprotein (BSP), runt-related transcription factor 2 (Runx2), osterix(Osx), osteocalcin (OCN), and alkaline phosphatase (ALP).(105) Runx2 is known to be expressed earlier in differentiation of osteoblasts, while Osx and OCN are known to be expressed later in differentiation.(52, 104, 106) Osteoblasts are normally in a quiescent state, and when they incorporate into the osteoid matrix they are referred to as osteocytes.(104) Osteocytes are the most mature cells of the osteoblastic lineage. In comparison to osteoblasts, osteocytes are smaller, have lower metabolic activity resulting in fewer organelles, and have a decreased alkaline phosphatase activity.(103)

Figure 1.1

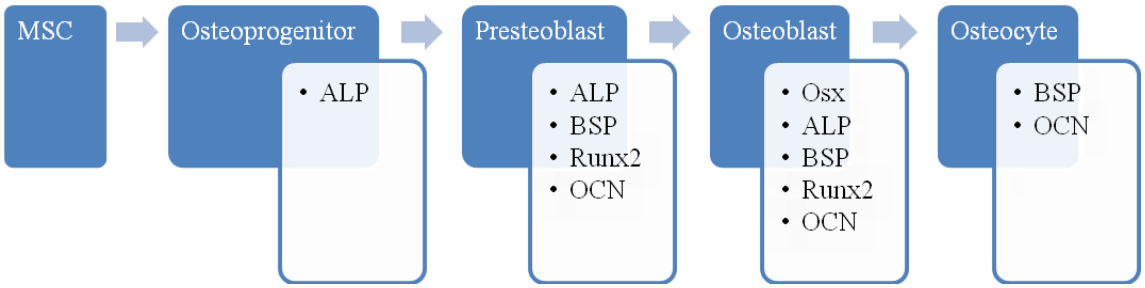

Figure 1.1 Expression of the addressed bone matrix proteins of the osteogenic cell lineage. Bone sialoprotein = BSP, Runt-related transcription factor 2 = Runx2, Osterix = Osx, Osteocalcin = OCN, and Alkaline phosphatase = ALP.

In vitro cultures of the cells from the osteoblastic lineage progress through a characteristic series of developmental stages.(107) The first recognizable stages are proliferation, then matrix development, and finally mineralization into nodules.(74) Different hormones and growth factors influence the *in vitro* development of osteoblastic cell lines at various stages.(104, 108) Osteoprogenitors may be influenced both in division and their ability to form nodules.(104) For example, the addition of glucocorticoids, most commonly dexamethasone, has been shown to increase the size and number of bone nodules,(109) as well as alkaline phosphatase enzymatic activity.(52) Ascorbic acid and β-glycerophosphate have been shown to enhance the mineralization of bone nodules.(107) The ability to consistently and repeatedly manipulate cells in the osteoblastic lineage *in vitro* is a critical prequisite to using these cells *in vivo*.(44)

1.5 Techniques to confirm the identity of cell lineages

Many molecular biological techniques have been implemented to confirm the cell lineages isolated and expanded in cell cultures. Expression of genes, detection of proteins of interest, and detection of cell surface markers are a few of the strategies being used.

1.5.1 Polymerase chain reaction

Polymerase chain reaction (PCR) is a method routinely employed in cell based research to identify the expression of specific genes.(110) The popularity of PCR is in part due to the fact that other techniques for detection of gene expression such as Northern and Southern Blots, require larger starting amounts of RNA and DNA respectively which can be tedious to generate in cell cultures.(111) PCR provides a way to exponentially replicate a small sample of a relatively short piece of DNA thus producing enough DNA that it can be tested and quantified.(112) PCR consists of 20-40 cycles through three different temperature steps known as thermal cycling. The first step takes place at 90-98^{o}C for 20-30sec and causes the denaturation of the DNA, and the destruction of hydrogen bonds between complimentary bases resulting in single stranded DNA (ssDNA). The next step occurs at 50-65^{o}C for 20-40sec and involves the annealing of the primers to their complementary DNA sequences. Finally the elongation step reaches 72^{o}C for 40-50secs, and nucleotides assemble along the ssDNA templates. Traditionally, agarose gel electrophoresis has been employed to visualize the PCR products and these are compared to a DNA ladder of known molecular weights. As this method for assessing amplicons is primarily qualitative in nature, real time quantitative PCR (qPCR) has been developed and is currently more often employed.(113)

1.5.2 Real time quantitative PCR

qPCR measures mRNA expression of the gene of interest compared to baseline expression of a reference (housekeeping) gene that is expressed at the same level in all cells. Normalizing expression levels of the gene of interest to this reference gene ensures that changes in gene expression are due to true biological differences, rather than differences in starting amounts of sample. Glyceraldehyde-3-phosphate dehydrogenase mRNA (GAPDH) is the housekeeping gene used in the experiments in this thesis because its expression levels were found by our preliminary work to have the least fluctuation when compared to other potential housekeeping genes such as βactin mRNA, and major histocompatibility complex 1. This finding of uniform GAPDH expression is consistent with previous qPCR studies in equine bone(114) and equine endometrium.(115)

Real time qPCR measures DNA, cDNA, or RNA expression of the gene of interest during the logarithmic phase of amplification instead of after the reactions are complete.(116-118) As the copies of DNA (cDNA) are amplified, a flourochrome, SYBR green, intercalates between the double-stranded DNA and fluoresces.(119, 120) This non-specific binding allows the DNA product to fluoresce to some degree, regardless of whether it is a contaminate gene or a gene of interest.(121, 122) For this reason, the melting point of each end product is measured and should be consistent with the known gene of interest's melting point if it is indeed pure.(119) The melting point is the temperature at which 50% of the DNA is denatured and it can be used to indentify PCR products. Impurities in a sample will cause it to have a separate melting point peak

from the other samples run in parallel.(123) The increasing intensity of fluorescence can be graphed and measured by a computer program as it takes place allowing for quantitative analysis of the reaction. A preset consistent florescence threshold is crossed as the DNA is amplified, and the number of cycles completed before reaching that threshold is called the C_T value. C_T levels are inversely proportional to the amount of target nucleic acid in the sample (ie the lower the C_T level the greater the amount of target nucleic acid in the sample).(117)

There are several methods to quantitate the mRNA levels that are obtained during qPCR. They are divided into absolute and relative quantification. Absolute quantification has been used to determine the absolute transcript copy number.(124-126) A standard curve is used to display the fold change in expression of the target and reference genes allowing for comparison.(127) While this gives quality information, it requires that you create a dilution curve for each reference and gene of interest on each plate. As this is tedious, and prone to dilution error that would render the data unusable, the standard curve method was not used in the research described in this thesis. The two most widely used relative quantification methods are the Pfaffl method and the comparative C_T method ($2^{-\Delta\Delta C_T}$). The Pfaffl method is a mathematical model that calculates the efficiency ratio of the reference gene to the gene of interest and therefore does not need a standard curve to do comparisons.(128) The comparative C_T method avoids the use of a standard curve as well as the calculation of gene efficiencies. In so doing it makes two assumptions that must be supported by the data. The first assumption is that the efficiency of the qPCR is close to 1 and the second is that the

efficiency of the reference gene and gene of interest are similar.(129) The efficiency was determined using bone derived MSCs as a positive control and a standard curve in preliminary work done before this thesis. As the efficiencies for qPCR assays used in the thesis research were ≥.98, the studies hereafter used the comparative C_T method to compare the gene expression between cell samples treated with osteogenic media and control samples of cells in standard media. This allowed the data to be presented as 'the expression of the gene of interest relative to the internal control or reference gene (GAPDH) in the treated sample (osteogenic media) compared with the untreated control (standard media).'(130)

1.5.3 Fluorescence-activated cell sorting, flow cytometry and immunofluorescence

The identification and enumeration of cells is a significant challenge in cell based research. Three biomedical techniques that rely on cell surface antigens and their interactions with antibodies are fluorescence-activated cell sorting (FACS), flow cytometry and immunofluorescence.(110) All three methods start by exposing a cell culture to fluorophore tagged antibodies for specific cell surface antigens. FACS and flow cytometry then suspend cells in a stream of fluid as a laser source is transmitted through the stream.(131) The specific light scattering and fluorescent characteristics of each cell allow them to be counted for flow cytometry, or to be counted and sorted one cell at a time, for FACS. Flow cytometry provides a quantitative reading of fluorescence from individual cells, and FACS provides this as well as a physical separation of cells of particular interest. To this end, the cells are tagged by antibodies

and fluorescent labels in preparation for the techniques thus changing their cell surface characteristics and impeding post-FACS/flow cytometric assays.(132) Despite this drawback, FACS is currently the standard for sorting populations and subpopulations of cells.(133) Immunofluorescence allows visualization of the fluorophore tagged cells, while they are still attached to plastic in cell culture, with a fluorescence microscope. The fluorescence is subjectively assessed and usually described as positive or negative.(134) All three of these techniques can be highly useful techniques when the cell surface markers of interest are well known.

1.5.3.1 Antibody selection for flow cytometry and immunofluorescence

When selecting an antibody for protein detection it is important to select a species whose antibodies have affinity for the species of interest.(135) There are now many reagent companies that specialize in providing both monoclonal (MAbs) and polyclonal (PAbs) antibodies for thousands of different proteins.(136) Antibodies that are induced by a variety of lymphocytes differentiating into plasma cells are known as polyclonal. MAbs are produced by a single B lymphocyte clone.(137) MAbs are much more difficult and time consuming to produce and are more expensive than PAbs. The affinity of PAbs for a certain protein is often much higher than that of MAbs because PAbs recognize multiple epitopes.(136) MAbs are easily deterred from attachment to their single epitope by conformational disturbances in the protein that effectively hide the epitope.(138) However, the purity of a specific antibody is higher in monoclonal preparations. Therefore, if searching for a specific protein, it is imperative to properly denature the protein to straighten the polypeptide chain and reveal the epitope allowing a

MAb to attach.(137) With these appropriate conditions, MAbs are much more specific for a certain epitope and have very little cross-reactivity.(139) MAbs' main advantage is their ability to be consistently produced from desired hybridoma while PAbs may change over time and are limited to the size and lifespan of the donor animal.(138, 140) Many laboratories have tried to create MAbs to surface markers specific to early osteoprogenitors or MSCs.(110, 135, 141) This is particularly challenging because no specific phenotypic features are known for absolute recognition of early osteoprogenitors at this time. In efforts to elucidate this phenotype as described in this thesis, it was hypothesized that sorting the MSCs would enable isolation of the cells with characteristic surface antigens. Then, identification of the cell surface markers would be less complicated and finally a study of their subsequent capacity for differentiation *in vivo* would be made possible.(84)

1.6 A need for immuno-tagless sorting

There is no single, specific stem cell marker, because stem cells express a wide variety of surface antigens.(135) There is also no consensus on definitive equine MSC clusters of differentiation cell surface marker (CD marker) expression, as there is for humans,(77) which complicates using a traditional immunological tagging system such as fluorescence-activated cell sorting (FACS) to sort equine stem cells.(110) Also, presence of surface markers does not denote that MSCs are in a completely undifferentiated state.(132) Due to a lack of reactivity between commercial monoclonal

antibodies and epitopes on equine cells, phenotyping has been incomplete to date.(142) Sorting the MSCs with a tag-less method may avoid these CD marker issues.

While as a whole, MSCs are considered multipotent, some pluripotent subpopulations of MSCs have been identified in humans(143-145) and rats,(146) and found to have different shapes, proliferation and differentiation abilities.(146, 147) This indicates the importance of the identification and isolation of the fraction of MSCs that proliferates and differentiates optimally for the application of interest.

1.7 Field-flow fractionation

Field-flow fractionation (FFF) refers to a group of bioanalytical techniques that have applications in the separation of bioanalytes ranging from proteins and nucleic acids to viruses, organelles and whole cells.(132) FFF techniques have been applied to cells to sort them by cell type,(148, 149) cell size,(150) for purification of cell culture,(151) to remove bacteria from bodily fluid,(152) and in conjunction with flow cytometry for cell characterization.(153)

1.7.1 Gravitational field-flow fractionation

Gravitational field-flow fractionation (GrFFF) is decribed as a type of FFF technique that relies on gravity to achieve sedimentation, and has been used to sort B and T lymphocytes,(154, 155) red blood cells,(156, 157) and MSCs from blood and bone marrow.(158, 159) MSCs differing in molar mass, size and surface antigens are driven by gravity into different velocity regions.(133) The cells are then carried

downstream through the channel at different speeds, and exit the channel after different retention times. The distribution of the cells into the various resulting fractions reveals the separation characteristics.(160) Traditional GrFFF systems then have a sedimentation step where an equilibrium between the gravitational field and the hydrodynamic forces of the transport fluid stream within the capillary channel is reached.(161) The resulting cell sedimentation in the system leads to cell adhesion to the wall of the capillary channel and cell–cell aggregation/stacking. The adherent nature of multipotent MSCs exacerbates this aggregation and necessitates non-equilibrium earth gravity assisted dynamic fractionation (NEEGA-DF), which skips the sedimentation step and increases cell recovery.(133)

1.7.2 Non-equilibrium GrFFF

Many aspects of the GrFFF technique make it ideal for use in cell culture. GrFFF allows for the expediting of MSC isolation by clearing other cells and contaminates from the early passage sample in a few hours instead of the weeks that can be spent performing the conventional method of adherence and detachment cycles. (158, 162) The shortening of this clean-up phase would be of great use in clinical application of stem cell therapy as it would shorten the return time on samples received for injured patients.(133) The GrFFF technology is similar to FACS in that it is a system to sort cells, but it is superior in several aspects. Currently FACS is the standard for sorting populations and subpopulations of cells.(133) The cells are tagged by antibodies and fluorescent labels in preparation for FACS thus changing their cell surface

characteristics and impeding post-FACS assays.(132) The use of immuno-tags for cell sorting is known to affect cell functionality.(160) GrFFF is a tag-less system of stem cell sorting that will avoid cell surface antigen augmentation of the MSCs.(133) GrFFF is much more economical than FACS as it can be assembled in the laboratory from instruments that most biotechnology laboratories own. GrFFF allows for the MSCs to be maintained under sterile conditions also allowing for further culture and expansion after fractionation.(132) A more attainable cell sorting system is needed because the vast expense and technical difficulty of a FACS system are prohibitive to many. To this end, the research described in this thesis used a non-equilibrium GrFFF separation mechanism to sort the MSCs.

1.7.3 Limitations of GrFFF

The main limitation of using non-equilibrium GrFFF as a sorting system is the low number of cells that can be fractionated per run.(133) Some solutions have been elucidated. One group set up two GrFFF channels in parallel to increase sorting throughput,(159) and placed a coating on the PVC in the GrFFF system which improved the sample returns.(163) They also changed the shape of the chamber to a tube and ran samples in tandem.(164)

1.8 Research objectives

The working hypothesis is that equine periosteum and skeletal muscle are equivalent, if not superior, multipotent sources of MSCs with osteogenic potential,

compared with results for the conventionally chosen donor tissues of fat and bone marrow. The primary goal of this project was to identify the ideal equine donor tissue and subpopulation as a source of MSCs to promote bone healing. To this end, the project had three objectives:

1. To confirm the ability to isolate, assess osteogenic capacities, and characterize mesenchymal stem cells (MSCs) from equine fat, bone marrow, periosteum, and muscle.

2. To sort the MSC populations, using non-equilibrium GrFFF, from two optimal tissues as determined in objective 1, and identify the MSCs subpopulations.

3. To identify those MSC subpopulations that express the highest osteogenic potential, and describe their phenotype.

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2. CHARACTERIZATION AND OSTEOGENIC POTENTIAL OF EQUINE MUSCLE TISSUE– AND PERIOSTEAL TISSUE–DERIVED MESENCHYMAL STEM CELLS IN COMPARISON WITH BONE MARROW– AND ADIPOSE TISSUE–DERIVED MESENCHYMAL STEM CELLS[1]

2.1. Abstract

The objective of this study was to characterize equine muscle tissue– and periosteal tissue–derived cells mesenchymal stem cells (MSCs) and assess their proliferation capacity and osteogenic potential in comparison with bone marrow– and adipose tissue–derived MSCs. Cells were isolated from left semitendinosus muscle tissue, right distomedial tibial periosteal tissue, bone marrow aspirates from the fourth and fifth sternebrae, and adipose tissue from the left tail head subcutaneous region. Mesenchymal stem cells were characterized on the basis of morphology, adherence to polystyrene plastic, trilineage differentiation, and detection of stem cell surface markers via immunofluorescence and flow cytometry. Mesenchymal stem cells were tested for osteogenic potential with osteocalcin gene expression via real-time PCR assay. Mesenchymal stem cell cultures were counted at 24, 48, 72, and 96 hours to determine tissue-specific MSC proliferative capacity. Equine muscle tissue– and periosteal tissue–derived cells were characterized as MSCs on the basis of spindle-shaped morphology, adherence to polystyrene plastic, trilineage differentiation, presence of CD44 and CD90 cell surface markers, and nearly complete absence of CD45 and CD34 cell surface markers. Muscle tissue–, periosteal tissue–, and adipose tissue–derived MSCs proliferated significantly faster than did bone marrow–derived MSCs at 72 and 96 hours. Equine muscle and periosteum are sources of MSCs. Equine muscle- and periosteal-derived MSCs have osteogenic potential comparable to that of equine adipose- and bone marrow–derived MSCs, which could make them useful for tissue engineering applications in equine veterinary medicine.

[1]Radtke CL, Nino-Fong R, Esparza Gonzalez BP, Stryhn H, McDuffee LA. Characterization and osteogenic potential of equine muscle tissue and periosteal tissue-derived mesenchymal stem cells in comparison with bone marrow and fat tissue-derived mesenchymal stem cells. Am J Vet Res. 2013;74:790-800.

2.2. Introduction

Catastrophic breakdown is a major source of wastage in racehorses, with as many as 67%-89% of deaths attributed to exercise-related injury.(1-3) Fractures account for 71% in UK, 88% in California, and 92.7% in Mid-west USA of fatal musculoskeletal injuries in racing Thoroughbreds.(1, 2, 4, 5) Because of their size and temperament, horses must bear weight on all limbs during the healing process. This leaves the surgical repair susceptible to failure because the constant loading of implants causes fatigue and ultimate mechanical failure of metal screws and plates.(6) The requirement for constant loading combined with the fact that fractures heal much slower in horses than in other veterinary species(7) leads to a guarded to poor prognosis for many equine fracture patients. If fracture healing rates could be increased, survival rates in affected horse may also increase, which would improve the animal welfare issue and make the initial fee for fracture fixation a more worthwhile investment to owners. Therefore, new methods to increase the rate of equine bone healing are needed and require research.

Studies addressing the healing of equine musculoskeletal injuries through MSC-based treatments, have shown that MSCs derived from amniotic membrane and bone marrow implanted into tendon lesions, have improved defect fill and decreased rate of re-injury.(8, 9) Mesenchymal stem cells are an option for clinical application because they can be effectively isolated and expanded with high efficiency.(10) Mesenchymal stem cells can be cryopreserved, they maintain their viability, and later can be induced to differentiate along multiple lineages.(11, 12) Although MSCs are being used clinically for certain musculoskeletal injuries, there are many unknown factors associated with their use, such as the ideal number of cells for transplantation, cell yield per gram of donor tissue, and ideal tissue source. Despite encouraging *in vivo* MSC treatment results

from equine soft tissue and joint injury studies such as the improved healing characteristics of femoropatellar cartilage lesions(13), meniscal lesions(13, 14), tendon and ligament lesions(8), little is known about the efficacy of equine MSCs for regeneration of bone *in vivo*.(15, 16)

Identification of the optimal source of MSCs with the best osteogenic potential may prove critical for moving the basic science research toward clinical cell-based treatments to promote bone healing.(15) Ideal tissue sources have been identified for human and rat MSCs on the basis of their intended use in vitro and in vivo,(17-20) and there is evidence that equine MSCs from bone marrow and fat have a high osteogenic potential, compared with that for MSCs from umbilical cord tissue and umbilical cord blood.(15, 21, 22)

Our laboratory group has identified periosteum and muscle tissues as sources of spindle-shaped, plastic-adherent cells able to undergo osteogenic differentiation.(23, 24) The working hypothesis of the group is that equine periosteum and skeletal muscle are equivalent, if not superior, multipotent sources of MSCs with osteogenic potential, compared with results for the conventionally chosen donor tissues of fat and bone marrow.

The purpose of the study reported here was to characterize equine muscle tissue– and periosteal tissue–derived MSCs, assess proliferative capacity of equine muscle- and periosteum-derived MSCs, and determine the osteogenic potential of equine muscle- and periosteum-derived MSCs in comparison with that of bone marrow– and adipose tissue–derived MSCs.

2.3. Materials and Methods

2.3.1. Samples

Ten adult horses (2 to 5 years old) were used for postmortem collection of samples of bone marrow, periosteum, skeletal muscle, and adipose tissue. The horses were donated to the Atlantic Veterinary College for reasons other than this study and were euthanized in accordance with protocols approved by the University of Prince Edward Island Animal Care Committee. All horses were sedated with xylazine (1.1 mg/kg, IV) and then euthanized via pentobarbital sodium injection (108 mg/kg, IV). All samples were collected immediately after horses were euthanized.

2.3.2. Bone marrow collection

A 10-cm-wide band overlying the sternum was clipped of hair. The skin over the fourth and fifth sternebrae was aseptically prepared, and a bone marrow biopsy needle (Carefusion, San Diego, CA) was used to obtain a bone marrow aspirate from the fourth sternebrae. The aspirate (9.5 mL) was collected into a 12-mL syringe that contained 2.5 mL of heparin (1,000 U/mL). Another aspirate was immediately obtained from the fifth sternebra in the same manner.

2.3.3. Adipose, muscle, and periosteum collection

The area left lateral to the base of the tail, the area superficial to the left semitendinosus and semimembranosus muscles, and the distomedial aspect of the right tibia were aseptically prepared. Skin incisions were made, and underlying tissues were harvested and approximate sizes and weights are as follows. A 24-cm^3 (3-g) section of

adipose tissue was harvested from the subcutaneous tissues over the gluteal muscles in the region of the base of the tail. A 9-cm^3 (6-g) section of muscle was dissected and harvested from the left semitendinosus muscle. A 4-cm^2 (0.5-g) section of periosteum from the medial surface of the proximal portion of the right tibia was elevated and harvested. The amount of sample collected was considered consistent with a clinically feasible biopsy specimen that would not result in adverse effects. The tissues collected were placed in α-MEM and transported to our laboratory.

2.3.4. Cell isolation from bone marrow

Cells were isolated from bone marrow via a centrifugation gradient technique. The samples were centrifuged in 50-mL tubes at 1,500 X *g* for 10 minutes. The buffy coat then was collected and placed into standard medium, which was composed of α-MEM supplemented with 10% fetal bovine serum, (PAA Laboratories Inc, Etobicoke, ON) L-glutamine (2mM), 10,000 U of penicillin and 10 mg of streptomycin/mL, and amphotericin B (250 µg/mL). This standard medium was maintained the same for the bone marrow and the other 3 tissues.

2.3.5. Fat, muscle, and periosteum cryopreservation

Fat, muscle, and periosteum were collected from each horse and placed in separate vials of chilled α-MEM solution. Tissues were processed within 24 hours after collection. Tissues that were not processed immediately were kept on ice and refrigerated at 4°C for 12 hours and then processed. Cold, sterile PBS solution was placed in Petri dishes to provide a moist environment for tissues subsequently cut into 1-cm segments. Tissue segments were placed into 2-mL cryovials and submerged in

freezing medium composed of 92.5% PBS solution and 7.5% dimethyl sulfoxide. The cryovials remained at room temperature (approx 20°C) for 30 minutes to allow the freezing medium to penetrate the tissue. The samples were then placed in closed-cell extruded polystyrene foam containers and stored in a –80°C freezer for a minimum of 24 hours. All samples were placed into a –196°C liquid nitrogen tank within 72 hours after processing.(25)

2.3.6. Fat, muscle, and periosteum cell isolation

Cells were isolated from fat, muscle, and periosteum by means of an enzyme digestion technique. Cryopreserved adipose and muscle tissues were warmed in a water bath (37°C) for approximately 5 minutes until the liquid was thawed. Tissue handling was performed via sterile technique in a biosafety cabinet. Each tissue was removed from the cryovials and placed in a 50-mL centrifuge tube that contained 25 mL of sterile PBS solution. The tissue was rinsed with PBS solution, weighed, and minced. Minced tissue was placed in centrifuge tubes that contained 10 mL of collagenase type I (Invitrogen, Toronto, ON) (2,000 units/mL); tubes then were mixed via a vortexer and placed in a 37°C incubator. The tubes were mixed via a vortexer every 20 minutes for 60 minutes. Once the tissue was digested, 10 mL of standard medium was added to the mixture to inhibit further enzymatic digestion. The cell suspension was filtered through a 100-µm filter and then a 70-µm filter and then was centrifuged at 377 x g for 10 minutes. Supernatant was removed, and the cell pellet was resuspended in standard medium. Viable cell numbers, as determined on the basis of 0.4% trypan blue dye exclusion, were counted with a hemacytometer. Viable adipose tissue and muscle cells were plated in T-75 flasks at a cell density of 33 X 10^3 cells/cm^2 in standard medium.

Cell cultures were maintained in a humidified incubator with 5% CO_2 and 95% air at 37°C.

Isolation of cells from the periosteum was similar to that of adipose tissue and muscle, except that the minced tissue was pretreated by incubation with type I collagenase (2,000 U/mL) for 10 minutes. The partially digested tissue was rinsed and treated by incubation with type I collagenase (2,000 U/mL) for an additional 160 minutes. Viable periosteal cells were plated in T-75 flasks at a cell density of 33 X 10^3 cells/cm^2 in standard medium.

2.3.7. Cell culture and cell passage

Cells adhered to the flasks by 24 to 48 hours (start of culture was designated as time 0); unattached cells were washed from the flasks with PBS solution. Medium was changed 3 times/wk. Cells were maintained in this environment until confluence, but culture did not exceed 9 days.

At each passage, cells were detached with 0.25% trypsin-EDTA. Cells were counted and viability determined with 0.4% trypan blue dye. First-passage cells from each tissue of each horse were used for a proliferation assay and to culture a second passage with higher cell counts. Second-passage cells from each tissue of each horse were used for a second proliferation assay and characterization assays at the various cell densities.

2.3.8. Characterization of MSCs and cell differentiation

Cells from each tissue were maintained for 1 week in standard medium. After this period, adherence to the flask and spindle-shaped morphology were confirmed.

Cells from each tissue from each of 3 horses were induced to differentiate into adipocytes, chondrocytes, and osteoblasts. Each of the 3 lineages was cultured in parallel with the same cells in standard medium as described. Light microscopy (Axiovert 40 CFL, Carl Zeiss Canada Ltd, Toronto, ON) digital images (Power shot G5, Canon, Mississauga, ON) were obtained at various times representative of the different morphologies. Histochemical analysis and morphology were used to confirm differentiation into the 3 lineages.

2.3.9. Adipogenic differentiation

Cells were seeded at a density of 12,000 cells/cm^2 into chamber slides (Becton-Dickinson, Falcon Bedford, MA). Cells initially were cultured in standard medium for 3 days. Thereafter, cells were incubated with an adipogenic induction medium (Dulbecco modified Eagle medium and F12, 3% fetal bovine serum, 10,000 U of penicillin, and 10 mg of streptomycin/mL, amphotericin B (250 µg/mL), biotin (Sigma, Oakville, ON) (33 µmol/L), pantothenate (Sigma, Oakville, ON) (17 µmol/L), insulin (1 µmol/L), dexamethasone (1 µmol/L), isobutylmethylxanthine (IBMX, Sigma, Oakville, ON) (0.5 mmol/L), rosiglitazone (Toronto Research Chemicals, Toronto, ON) (5 µmol/L), and 5% rabbit serum (Invitrogen, Toronto, ON)) for 2 days. The same medium without isobutylmethylxanthine and rosiglitazone then was used to maintain the adipocyte cell culture until day 7, when the cells were fixed for 20 minutes in 10% formalin at room temperature and stained for neutral lipid accumulation with oil red O to indicate adipogenic differentiation.(21)

2.3.10. Chondrogenic differentiation

Cells were seeded at a density of 500,000 cells/15 mL in polypropylene conical tubes and were then centrifuged (500 X *g* for 5 minutes) into pellets, which were supplemented with a chondrogenic differentiation medium (Hams 12; dexamethasone (10^{-7}M); culture supplement containing bovine insulin, transferrin, selenious acid, linoleic acid, and BSA, (ITS+1, Sigma, Oakville, ON) 5% fetal calf serum, 10,000 U of penicillin and 10 mg of streptomycin/mL, amphotericin B (250 µg/mL), ascorbic acid (50 µg/mL), and recombinant human transforming growth factor-β 1 (rhTGF-beta 1, Millipore, Temecula, CA) (11 ng/mL)). Pellet cultures were maintained for 21 days. Pellet cultures were performed in parallel with standard medium and chondrogenic medium, with no growth factor as a control culture. After culture for 21 days, differentiated pellets were fixed in 10% formalin for 24 hours, dehydrated in a graded series of ethanols, and embedded in paraffin. A microtome (Leica RM 2245, Leica Microsystems, Richmond Hill, ON) was used to make sections (thickness of 5 µm) that were then stained with Alcian blue (pH, 1.0) for the detection of cartilage-specific proteoglycans to confirm chondrogenic differentiation.(26)

2.3.11. Osteoblastic differentiation

Cells were seeded at a density of 12,000 cells/cm^2 into chamber slides. Cells were supplemented with an osteogenic induction medium (α-MEM, 5% fetal calf serum, 10,000 U of penicillin, and 10 mg of streptomycin/mL, amphotericin B (250 µg/mL), ascorbic acid (50 µg/mL), dexamethasone (10^{-8}M), and β-glycerophosphate (10mM)). Cultures were maintained for 7 days and then fixed for 20 minutes in 10% formalin at room temperature.(24) Cultures were then stained with von Kossa stain(26) for the detection of calcium and with the substrate naphthol AS MX-PO_4 and Red Violet LB

salt(27) for the detection of alkaline phosphatase to confirm mineralization and osteoblastic differentiation.

2.3.12. Immunofluorescent analysis for MSC surface markers

Cells from each tissue from each of 3 horses were plated at 2,500 cells/cm^2 in standard medium. Cells were then incubated at 37°C for 24 hours in a humidified incubator at 5% CO_2 and 95% air. Culture medium was removed and cells were washed twice with PBS solution. Cells were fixed in 4% paraformaldehyde (pH, 7.4) for 15 minutes. Paraformaldehyde was removed and cells were washed twice with PBS solution. Cells were blocked by incubation with 1% BSA in PBS solution at room temperature for 1 hour. The blocking solution was then removed and the remaining steps were conducted in a dark room. One microliter of antibodies (CD34 (AbD Serotec, Raleigh, NC), CD44 (Abcam Inc, Cambridge, MA), CD45 (AbD Serotec, Raleigh, NC), CD90 (Accurate Chemical and Scientific Corp, Westbury, NY), CD105 (Chemicon International, Billerica, MA), and CD146 (Millipore, Billerica, Mass); FITC was the fluorochrome for CD34, CD44, and CD45 and Alexa Fluor 88 was the fluorochrome for CD 146) diluted in 1% BSA in PBS solution were added to the cells, and plates were incubated overnight at 4°C. The next day, cells marked with the CD90 antibody were washed twice with PBS solution, and FITC-labeled secondary antibody (Goat anti-mouse IgG FITC-labeled antibody, Cedarlane Laboratories Ltd, Burlington, ON) diluted in 1% BSA in PBS solution was added. Cells were then maintained at room temperature for 1 hour. All cells were covered with PBS solution and allowed to sit for 5 minutes at room temperature in dark conditions; this process was repeated with fresh PBS solution 3 times. A nucleic acid stain (Hoechst 33258, Invitrogen, Toronto, ON) (1µM of stock

solution/10 mL of distilled water) was added to all cells; cells were incubated for 1 minute and then washed once with PBS solution. Cells remained in PBS solution, and digital images were obtained immediately. Cells from each of the tissues were evaluated for positive results for MSC surface markers.

2.3.13. Flow cytometric analysis of MSC surface markers

Cultured and expanded cells from the second passage of each of the 4 tissues (bone marrow, fat, periosteum, and muscle) from 1 horse were used for the flow cytometric analysis. The amount of antibody was optimized with a cytometer (FACSAria flow cytometer, BD Biosciences, Mississauga, ON).

Cells were washed with PBS solution and then incubated for 15 minutes in a humidified incubator at 5% CO_2 and 95% air at 37°C with a mixture of versene (Invitrogen, Toronto, ON) and trypsin (5:1). This detachment method yielded the highest values for viability (up to 95% after 8 hours). The reaction was stopped with an equal amount of standard medium. The cell suspension was centrifuged (377 X *g* for 10 minutes), and the pellet then was resuspended and washed in ice-cold 1% BSA in PBS solution. The cell suspension was again centrifuged (377 X *g* for 10 minutes), and the resulting pellet was resuspended in ice-cold 1% BSA in PBS solution, stained with trypan blue to determine viability, and counted for flow cytometric analysis.

One million cells per sample were labeled. Sample 1 was unstained and served as a negative control sample. Samples 2 to 5 were labeled with validated(28, 29) antibodies (CD45, CD44, CD90, and CD34, respectively). Sample 6 was labeled with a combination of 45 allophycocyanin (AbD Serotec, Raleigh, NC), 44 R-phycoerythrin

(Biolegend, San Diego, CA), and 90 FITC (Accurate Chemical, Westbury, NY). Sample 7 was labeled with a combination of 34 allophycocyanin, 44 phycoerythrin, and 90 FITC. One million cells were collected and centrifuged (377 X *g* for 10 minutes), and primary antibodies were added in 1% BSA in PBS solution (Table 2.1). Samples were placed on ice and incubated for 45 minutes; samples then were washed in ice-cold 1% BSA in PBS solution and centrifuged (377 X g for 10 minutes). The washing and centrifugation steps were repeated 3 times. Cells were stored at 4°C until flow cytometric analysis. The secondary antibody for CD90 was diluted in 1% BSA in PBS solution and incubated on ice for 30 minutes and then washed in ice-cold 1% BSA in PBS solution and centrifuged (377 X *g* for 10 minutes). The washing and centrifugation steps were repeated 3 times. Cells were stored at 4°C until flow cytometric analysis (Aurum total RNA mini kit, Bio-Rad Laboratories, Hercules, CA).

Table 2.1 Characteristics of antibodies used for flow cytometric analysis of MSC surface markers.

CD marker	Fluorochrome	Emission wavelength (nm)	Excitation wavelength (nm)
CD34	Allophycocyanin	660	650
CD44	Phycoerythrin	667	496
CD45	Allophycocyanin	660	650
CD90	NA	NA	NA
Goat anti-mouse IgM	FITC	520	494

NA = Not applicable.

2.3.14. Proliferation assay and MSC yield

Cells from each tissue from each of the 10 horses were plated (in triplicate) in 35-mm wells at 3,000 cells/cm^2. The cells were detached with trypsin and counted at 24, 48, 72, and 96 hours with a hemacytometer to determine the proliferation rate and doubling time. The procedure was repeated for the first and second passage of each tissue.

At the first passage, data were collected from the 10 horses to determine the mean MSC yield (MSCs obtained per gram of tissue) for bone marrow, adipose tissue, muscle, and periosteum. The volume of bone marrow and weight of the 3 other tissues were recorded during the isolation and culture procedures. Equine bone marrow has a mass density of 1 g/mL.(25) Therefore, the bone marrow volumes were converted to weights for ease of comparison with results for the other 3 tissues.

2.3.15. Osteogenic assay

Cells from the second passage of all 10 horses and all 4 tissues were seeded in 6-well plates at 200 cells/cm^2. Half of the wells were induced with osteogenic medium, and the other half were maintained in standard medium to serve as control cultures. Growth of one group of the paired cultures was stopped on day 7, and the other was stopped on day 10. Total RNA was extracted (Aurum total RNA mini kit, Bio-Rad Laboratories, Hercules, CA) from the cells. The cDNA was synthesized from total RNA via a cDNA synthesis kit (iScript cDNA synthesis kit, Bio-Rad Laboratories, Hercules, C). Primers derived from the coding regions of osteocalcin were as follows: forward, 5'-CTGGGCCAGGACTCCGCATCT-3'; and reverse, 5'-AGCCAGCTCGTCACAGTCTGGGTTG-3'. Expression of the osteocalcin gene was

quantified via real-time PCR assay with a mix (iQ SYBR Green Supermix, Bio-Rad Laboratories, Hercules, CA). The PCR assay was performed on a thermal cycler (Rotorgene-6 RG 3000, Corbett Research, Montreal, QC). Cycling conditions were as follows: 95°C for 5 minutes; 35 cycles of 95°C for 15 seconds, 56°C for 30 seconds, and 72°C for 45 seconds; and melting from 55° to 99°C. Nuclease-free water instead of cDNA was used as a negative control sample. Gene expression was determined via the comparative cycle threshold (ΔΔCT) method.(30) The housekeeping gene glyceraldehyde 3-phosphate dehydrogenase was used to normalize the expression of each osteocalcin gene.(31, 32)

2.3.16. Statistical analysis

Statistical analysis of proliferation data was conducted with a linear mixed model on logarithmically transformed outcomes with fixed effects of tissues, passages (first and second), and time (24, 48, 72, and 96 hours) as well as all interactions and random effects of horses, tissues within horses, passages within tissues, and sets of triplicates for each of the time points. Pairwise comparisons between tissues within time points underwent a Holm adjustment for multiple testing.(33) An additional analysis with an assumed linear effect of time was used to estimate doubling times for each of the tissues.

Statistical analysis of osteocalcin gene expression measured with a real-time PCR assay was conducted via a 2-way ANOVA with effects of tissues and horses, after square root transformation of the normalized cycle threshold values to comply with model assumptions $P < 0.05$. Data for days 7 and 10 were analyzed separately. Tissues were compared for their expression of osteocalcin with that of the standard medium via

t-tests on the basis of least squares means. Pairwise comparisons among tissues were conducted with the Tukey method P<0.05.

Statistical software was used for the analysis of proliferation data (SAS, version 9.2, SAS Institute Inc, Cary, NC) and osteocalcin data (Minitab, version 16, Minitab Inc, State College, PA). Significance was set at values of $P < 0.05$.

2.4. Results

2.4.1. Characterization of MSC morphology, adherence, and confluence

Cells isolated from all 10 horses and all 4 tissues developed a spindle-shaped morphology and readily adhered to polystyrene plastic. The MSC cultures derived from muscle, periosteum, and adipose tissue became 80% to 100% confluent within 6 to 8 days after initial seeding of flasks in the first passage. However, MSC cultures derived from bone marrow consistently required longed to achieve confluence and only achieved 45% to 75% confluence during this time.

2.4.2. Differentiation

Cells isolated from 3 horses and all 4 tissues were capable of trilineage differentiation (Figure 2.1). Cells cultured in adipogenic differentiation medium for 4 days had positive results for oil red O staining of lipid droplets. Cells cultured in standard medium did not develop lipid droplets and lacked staining with oil red O. Pelleted MSCs cultured in chondrogenic differentiation medium for 21 days had cells within lacunae in Alcian blue–stained material. Cells cultured in standard medium did not have lacunae and lacked staining with Alcian blue.

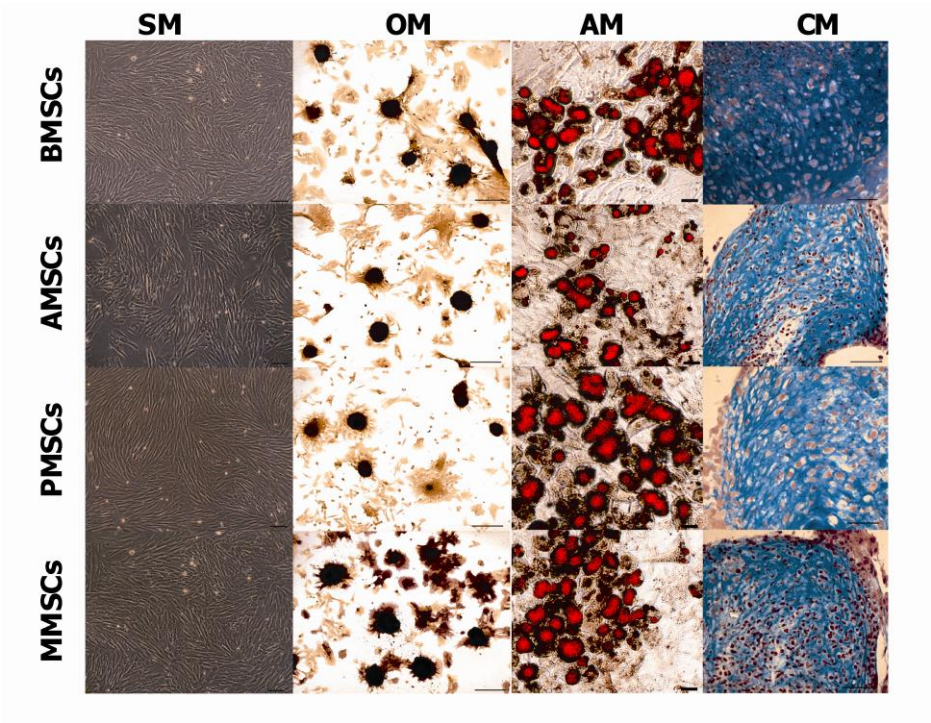

Figure 2.1—Representative photomicrographs of histochemical staining for MSCs cultured from equine bone marrow (BMSC), adipose tissue (AMSC), periosteum (PMSC) and muscle (MMSC). Standard medium (SM) is unstained, osteogenic medium (OM) is stained with von Kossa stain, adipogenic medium (AM) is stained with oil red O, and chondrogenic medium (CM) is stained with Alcian blue. Bar = 200 µm.

Cells cultured in osteogenic differentiation medium for 7 to 10 days formed bone nodules based on positive results of alkaline phosphatase and calcium specific stains. Cells cultured in standard medium did not develop nodules and lacked staining for alkaline phosphatase and calcium.

2.4.3. Immunofluorescent analysis of CD markers

Cells isolated from 4 horses and all 4 tissues cultured via standard conditions in the first passage strongly expressed the cell surface antigen CD90 and weakly expressed

CD44, as determined on the basis of immunofluorescence. None of the isolated cells had immunofluorescence for CD45, CD34, CD146, or CD105 (Figure 2.2).

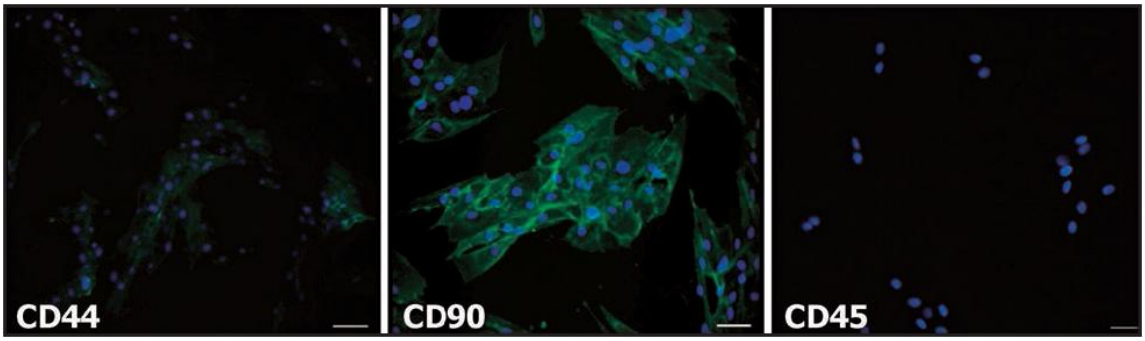

Figure 2.2—Representative images of immunofluorescence labeling of muscle-derived MSCs cultured from horses and stained for cell surface markers. Notice the weak staining for CD44, strong staining for CD90, and absence of staining for CD45. Bar = 200 µm.

2.4.4. Flow cytometric analysis

Cells isolated from 1 horse and all 4 tissues that were cultured via standard conditions in the second passage expressed high amounts of the cell surface antigens CD90 and CD44 and expressed extremely low amounts of CD45 and CD34, as determined on the basis of flow cytometric data (Figure 2.3; Table 2.2).

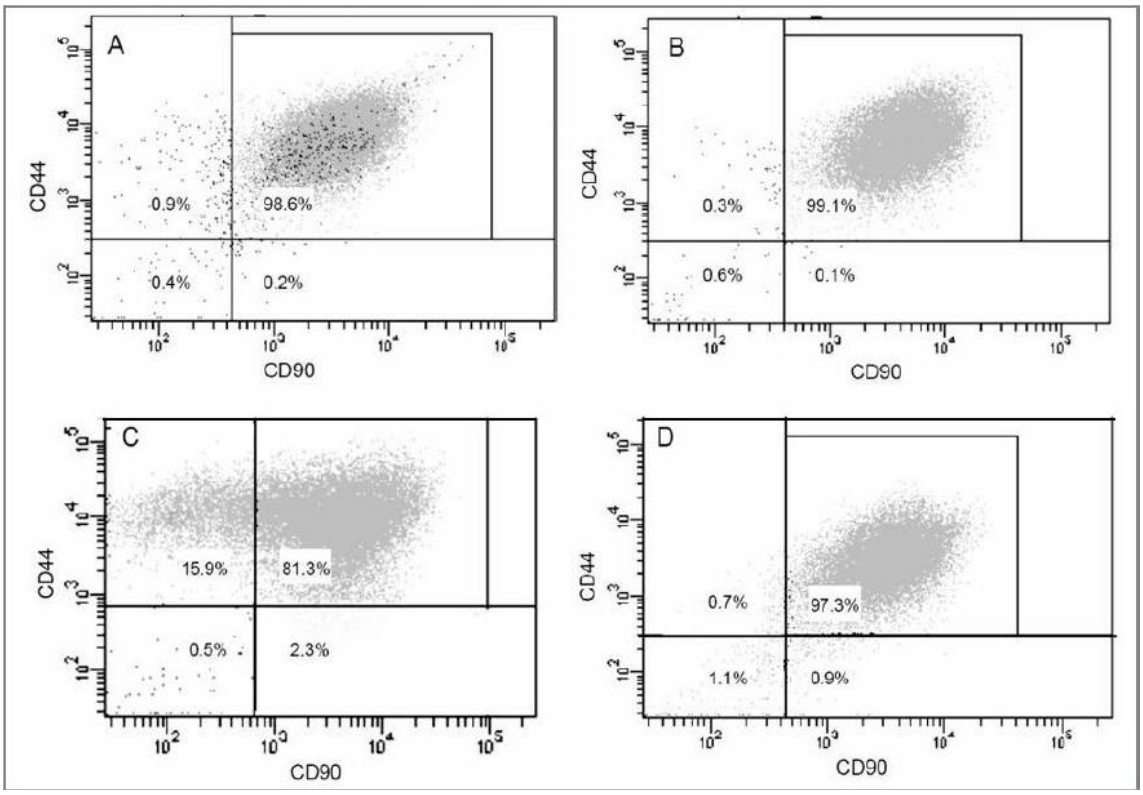

Figure 2.3—Results of flow cytometric analysis of MSCs cultured from equine muscle (A), periosteum (B), bone marrow (C), and adipose tissue (D) and stained for detection of CD90 and CD44 expression. Percentage values indicate the number of cells within each quadrant. The upper right quadrant contains +CD44 and +CD90 cells. The upper left quadrant contains +CD44 and –CD90 cells. The lower left quadrant contains –CD44 and –CD90 cells. The lower right quadrant contains –CD44 and +CD90 cells. The fluorescence intensity (arbitrary units) is depicted.

Table 2.2—Expression and coexpression of CD markers for each tissue obtained from 1 representative horse.

CD marker	Fat	Bone marrow	Periosteum	Muscle
CD90+	95.5	80.2	98.2	97.8
CD44+	97.5	97.1	99.2	99.1
CD34+	7.8	2.8	3	2.7
CD45+	8.6	1.2	1.6	2.3
CD90+ CD44+ CD45+	4.9	1.5	1.5	1.9
CD90+ CD44+ CD34+	3.3	2.4	1.2	2.2
CD34+ CD90– CD44–	0.3	0	0	0.4
CD45+ CD90– CD44–	0.5	0	0	0

Values reported are percentages.
+ = Positive results for the indicated marker. – = Negative results for the indicated marker.

2.4.5. Proliferation rate

Analysis of the logarithmically transformed MSC counts revealed a significant ($P < 0.001$) interaction between tissues and times, whereas no significant effects were detected for passages (including interactions). At 72 and 96 hours, muscle-, periosteum-, and adipose-derived MSCs proliferated significantly faster than did bone marrow–derived MSCs (Figure 2.4). Assuming a log-linear effect of time (equivalent to assuming a constant doubling rate) yielded estimated doubling times for MSCs derived from bone marrow (27.3 hours), periosteum (15.0 hours), muscle (13.8 hours), and adipose tissue (16.2 hours). All pairwise comparisons between doubling times yielded significant results, except for periosteum-derived MSCs, compared with results for muscle- or adipose-derived MSCs. The model without assumed linearity of time yielded a

significantly (P = 0.01) better fit for the data and was thus preferred for reporting purposes.

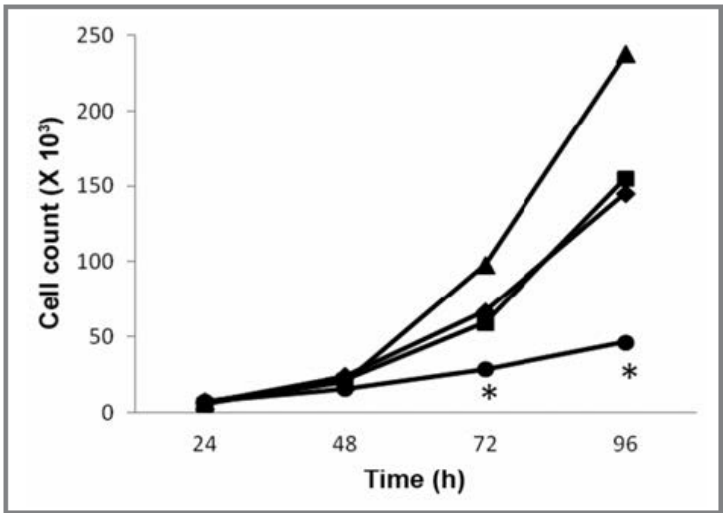

Figure 2.4— Graph of model-based estimated median cell counts of MSCs versus time after start of culture for MSCs derived from equine muscle (▲), periosteum (■), adipose tissue (♦), and bone marrow (●). Start of MSC culture was designated as time 0. *Within a time point, value for bone marrow differs significantly (P = 0.01) from the values for all other tissues.

2.4.6. MSC yield

Periosteum provided a higher MSC yield than did the other 3 tissues. Periosteum yielded a mean of 30.3 million cells/g of tissue, muscle yielded 642,000 cells/g of tissue, adipose tissue yielded 1.7 million cells/g of tissue, and bone marrow yielded 83,000 cells/g of tissue.

2.4.7. Real-time PCR assay for osteocalcin expression

Osteogenic capacity determined on the basis of gene expression of osteocalcin was measured in all 4 tissues from all 10 horses. Muscle-, periosteum-, adipose-, and

bone marrow–derived MSCs all had significantly higher osteocalcin expression on day 7 after differentiation with osteogenic medium than did the control samples cultured in standard medium. There was no significant (P = 0.17) difference in osteocalcin expression among the tissues. There were no significant differences between differentiated and nondifferentiated cultures of MSCs on day 10 (Figure 2.5).

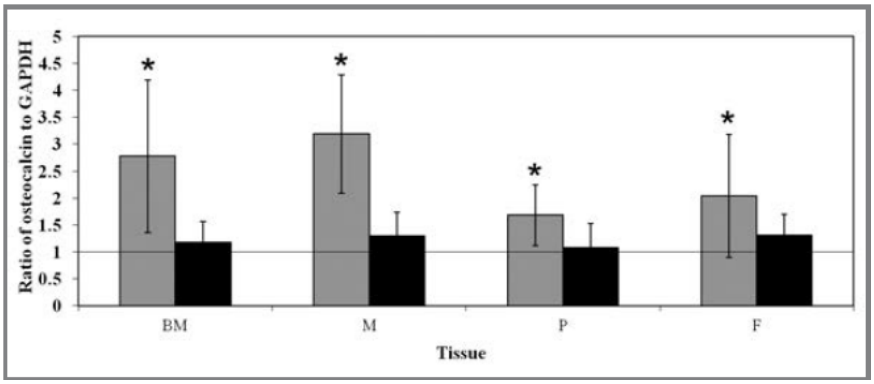

Figure 2.5—Mean ± SD results of nontransformed data for real-time PCR assay of osteocalcin gene expression for MSCs derived from 4 tissues obtained from 10 horses and cultured for 7 (gray bars) and 10 (black bars) days. *Within a tissue, value differs significantly (P < 0.05) from the baseline number of MSCs cultured in standard medium (horizontal black line). GAPDH = Glyceraldehyde 3-phosphate dehydrogenase. BM= Bone marrow; M= Muscle; P= Periosteum; F= Fat.

2.5. Discussion and Conclusions

Analysis of results of the present study confirmed that cells derived from equine muscle and periosteal tissues can be characterized as MSCs, equine muscle- and periosteum-derived MSCs have superior proliferative capacity to that that of bone marrow–derived MSCs, and equine muscle- and periosteum-derived MSCs have osteogenic potential comparable to that of equine adipose- and bone marrow–derived

MSCs. In this study, equine muscle and periosteal tissues were sources of MSCs, as determined by morphology, adherence to plastic, trilineage differentiation, and detection of stem cell surface markers with immunofluorescent and flow cytometric analyses.

Muscle and periosteum are good sources of MSCs in rats(18) and dogs,(25) but only muscle has been validated as a source of MSCs in horses.(34) In humans, the importance of muscle-derived MSCs(35) and periosteum-derived MSCs in bone repair (36, 37) have been reported. Therefore, it appears reasonable that muscle and periosteum may be useful sources of MSCs in horses as well. Muscle-derived MSCs have been isolated from horses and evaluated for potential tendon differentiation,(34) and periosteum-derived MSCs have been isolated from horses and evaluated for their potential osteogenic differentiation.(24) However, muscle- or periosteum-derived MSCs have not been thoroughly characterized as MSCs.

We used post-mortem collected tissues due to the fact we were collecting multiple tissues from multiple animals, and the animals available for the collection of tissues were horses admitted for elective euthanasia. Cell viability from tissues obtained immediately after death had viability approaching 90% and maintained good viability throughout the study similar to a previous study in dogs where postmortem tissues were used.(25)

The rationale for cryopreserved whole tissue sections was to preserve samples for later stem cell recovery. Immediate cryopreservation of tissues was considered more practical than direct primary isolation of stem cells, which requires additional equipment and personnel. Cryopreservation techniques may be advantageous for banking of specimens from which MSC cultures are not immediately needed. This is supported by three studies(38-40) in humans in which found that cells isolated from tissue processed

and frozen with cryopreservation medium and subsequently thawed maintained morphological and developmental competence and had MSC-hallmark trilineage differentiation with appropriate culture conditions. The buffy coat of the bone marrow does not survive cryopreservation procedures well and was therefore cultured immediately after collection from the horses of the present study.

Equine MSCs derived from bone marrow and adipose tissue MSCs have been characterized on the basis of morphology, adherence to plastic, trilineage differentiation, and CD markers,(41-43) and results of the present study confirmed those findings. In addition, cells isolated and expanded from muscle and periosteal tissues were characterized as MSCs on the basis of the identical criteria accepted for equine MSC characterization.

Characterization with immunofluorescent staining revealed strong expression of the cell surface antigen CD90 for cells of all tissues, which indicated the cells were MSCs. Slight weakness of CD44 expression could have been attributable to sensitivity to the proteolytic action of trypsin, which is the agent most commonly used to detach cells during cell culture.(44) Another explanation of the slightly weaker staining for CD44 is that direct immunolabeling was used to detect CD44, but an indirect immunolabeling method, which is more sensitive, was used to detect CD90.(45) In addition, there can be variations in expression of cell surface markers on the basis of differences in culture times and isolation techniques.(46, 47) None of the isolated cells stained for CD45, CD34, CD146, or CD105, which is consistent with findings for equine MSC surface markers in another study.(42)

Characterization with immunophenotyping also revealed that cells from periosteum and muscle as well as those from fat and bone marrow could be

characterized as MSCs. Although there is currently no definitive consensus for expression of CD markers of equine MSCs,(28) findings in the present study match those of other studies that were positive for the expression of cell surface markers CD90 and CD44(48, 49) and low or negative expression of CD34 and CD45.(42)

To our knowledge, this is the first study conducted to characterize equine muscle- and periosteum-derived MSCs as defined on the basis of dual expression of CD44 and CD90 and extremely low expression of CD34 and CD45. Interestingly, for each of the 4 tissues, the percentages of cells with dual expression of CD90 and CD44 were extremely close to those with single positive staining for each cell surface marker, and the relatively high percentages indicated a reasonably pure population of MSCs from each tissue. Bone marrow–derived MSCs had a lower percentage of dual staining cells in all 4 tissues, which confirmed that the concentration of MSCs was lower in bone marrow than in the other 3 tissues.

Analysis of data from the present study revealed that bone marrow–derived MSCs proliferated slower than did muscle-, periosteum-, and adipose-derived MSCs. This is consistent with previous findings that muscle-derived cells yield greater cell culture numbers in a shorter time than do bone marrow–derived cells.(21, 34)

Bone marrow may not be the optimum tissue for use in bone healing when time to culture clinically useful numbers of autogenous cells is considered. Bone marrow–derived MSCs also senesce much earlier than do other MSCs of horses.(50) The slow proliferation of bone marrow–derived MSCs could have been attributable to the low number of proliferative cells in bone marrow aspirates. On the basis of a CFU fibroblast assay, the frequency of MSCs in the mononuclear cell fraction of equine marrow is reported to be 1 in 4.2 X 10^3 cells and to differ among horses by 10-fold.(43) The small

fraction of proliferative cells among the total cell isolates could be to blame for the 1 to 2 weeks of extra expansion time needed for bone marrow–derived MSCs over the expansion time needed for other sources of MSCs.(51, 52) This points to the need for a cell-sorting method to isolate this small proliferative fraction of cells before culture, which would aid in the removal of contaminant cells that physically impede MSC adherence and thereby hasten the expansion process.

Periosteum provided a higher MSC yield than did the other 3 tissues. Periosteum yielded a mean of 365 times as many MSCs/g of tissue then did bone marrow at the end of passage 0. Although the exact dose of MSCs for various injuries has yet to be determined, there is evidence that MSC effect is a dose-dependent phenomenon.(53-55) It is clinically important to use stem cell sources that are extremely proliferative because treatment is dependent on the number of cells in cultures, and up to 70 million osteoblasts may be required to generate 1 cm^3 of bone.(56)

In addition, ease of harvest, quantity of donor tissue available, and morbidity at the donor site are clinically important issues. The general requirement for treatment with stem cells is that the cells be from a readily available source and there is low morbidity associated with donor harvest. Muscle tissue, which can be readily harvested (similar to adipose tissue), meets these criteria. Harvest of bone marrow from the sternebrae is more difficult and can be associated with the risk of entering the thoracic cavity and potentially puncturing the pericardium when attempting aspiration from the sternum.(57, 58) The temperament of the horse as well as the expertise of the veterinarians harvesting the bone marrow are risk factors associated with this method of harvest. Methods to optimize and improve the safety of bone marrow harvest by ultrasound guidance of aspirate needle into the 4-6^{th} sternebrae have recently been

recommended and shown effective.(59) Bone marrow can also be harvested from the tuber coxae to avoid the risk associated with aspiration from the sternum; however, a recent study(60) found reduced numbers of MSCs for aspiration of the tuber coxae, compared with results for aspiration of the sternum in middle-aged horses. Periosteum was relatively easy to harvest as well, but horses would likely need to be anesthetized. Harvest would be clinically feasible at the time of fracture repair because it only requires removal of a 4-cm^2 section of periosteum from the fracture site or a remote donor site.

In the present study, we determined that equine muscle and periosteal tissues are donor sources of MSCs that have osteogenic potential for bone healing. It is unlikely that a single donor source of MSCs will be superior for regeneration of tissue from all different germ layers.(61) In one in vitro study,(21) adipose-derived MSCs required longer than did bone marrow–derived MSCs to undergo osteogenic induction, and investigators in another in vitro study(15) found that bone marrow–derived MSCs required longer to undergo osteogenic induction and had more osteogenic potential than did adipose-derived MSCs. Therefore, there is a need for research on which donor tissues are most suitable for use in bone healing and we plan to address this in the future.

Real-time PCR assay for osteocalcin expression was used in the present study to confirm osteoblastic differentiation.(62, 63) Osteocalcin is an abundant noncollagenous, hydroxyapatite-binding protein found in bone that is commonly measured and is a specific marker for the osteoblastic stage of osteogenesis.(19, 35) Mesenchymal stem cells from all 4 tissues could be induced to differentiate into the osteoblastic lineage, as indicated by an increase in osteocalcin expression measured on day 7. Mesenchymal stem cells from day 10 had no significant differences between differentiated and nondifferentiated cultures, which indicated a decrease in osteocalcin expression between

day 7 and 10, which is consistent with a temporal sequence of osteogenic differentiation.(64)

One of the limitations of the present study was the use of a hemacytometer for cell counts. Automated cell counters may have a lower error margin, but we adhered to research protocols that involved the use of hemacytometers.(21, 37, 41) All counts were performed in triplicate for each sample to improve accuracy. Another limitation of the study was that counts of MSCs per gram of tissue did not address heterogeneity of the tissue. However, it is a repeatable and acceptable method(25, 65, 66) to measure and compare cell yield from tissues because a weight measurement is more easily made and more accurate than is a size measurement. Finally, these measurements were paired with clinically feasible sizes of biopsy specimens for practical application. Another limitation of this study was the use of a single osteoblastic marker. Evaluation of the expression of additional genes as osteoblast markers may have highlighted differences in osteogenic potential among tissues, considering that no significant differences were detected with use of 1 marker.(67) Because the focus of this study was to confirm osteogenic differentiation as part of trilineage differentiation, osteocalcin was used as the sole marker.(62, 63) Finally, although there was no significant difference ($P = 0.17$) in osteocalcin expression among the 4 tissues of the 10 horses, it is possible that an increased number of horses may yield data in which the differences are significant.

In humans, periosteum and muscle clearly are potent sources of bone-forming cells for use in orthopedic repair.(35, 36) To our knowledge, the study reported here is the first to confirm osteocalcin expression in equine muscle- and periosteum-derived MSCs, which indicates their osteogenic potential. The characterization of muscle- and periosteum-derived MSCs broadens the choices available to clinicians who use MSCs in

cell-based treatments, and MSCs from these tissues show much promise for future application in cell-based treatment for use in bone healing in horses.

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3. APPLICATION OF A NOVEL MESENCHYMAL STEM CELL SORTING SYSTEM FOR EQUINE MESENCHYMAL STEM CELLS[1]

3.1. Abstract

The objective of this study was to validate non-equilibrium gravitational field-flow fractionation (GrFFF), an immunotag-less method of sorting mesenchymal stem cells (MSCs) into subpopulations, for use with equine muscle tissue-derived, periosteal tissue-derived, bone marrow-derived, and adipose tissue-derived mesenchymal stem cells. Cells were isolated from left semitendinosus muscle tissue, periosteal tissue from the distomedial aspect of the right tibia, bone marrow aspirates from the fourth and fifth sternebrae, and left supragluteal subcutaneous adipose tissue of 6, adult horses. Aliquots of 800 $x10^3$ MSCs from each tissue source were separated and injected into a ribbon-like capillary device by continuous flow (GrFFF proprietary system). Cells were sorted into 6 fractions and absorbencies (OD) were read. Six fractions from each of the 6 aliquots were then combined to provide pooled fractions that had high enough cell numbers to seed at equal concentrations into assays. Equine muscle tissue-derived, periosteal tissue-derived, bone marrow-derived, and adipose tissue-derived mesenchymal stem cells were consistently sorted into 6 fractions that remained viable for use in further assays. Fraction one had a more cuboidal morphology in culture when compared to the other fractions. Statistical analysis of the fraction absorbencies (OD) revealed a significant difference (P-value of <0.05) when fraction 2 and 3 were compared to fractions 1, 4, 5, and 6. Non-equilibrium GrFFF is a valid method for sorting equine muscle tissue-derived, periosteal tissue-derived, bone marrow-derived, and adipose tissue-derived mesenchymal stem cells into subpopulations that remain viable, securing its potential for use in equine stem cell applications and veterinary medicine.

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3.2. Introduction

The need for methods of cell sorting that are less expensive, more practical, and less compromising of cell viability are an important next step in both equine and human cellular based therapy.(1, 2)(Chapter 2) Currently fluorescence-activated cell sorting (FACS) is the standard for sorting populations and subpopulations of cells.(2) The specific light scattering and fluorescent characteristics of each cell allow them to be sorted one cell at a time. While it provides a quantitative reading of fluorescence from individual cells as well as physical separation of cells of particular interest, the cells are tagged by antibodies and fluorescent labels in preparation for FACS, thus changing their cell surface characteristics and impeding post-FACS assays.(3) The vast expense and technical difficulty of a FACS system, the dedicated technical support to operate it, and the reagents required are prohibitive to many, creating a need for more readily accessible options in cell sorting.

The lack of a definitive consensus on equine MSC CD marker expression is another complicating factor with using an immunological tagging system to sort equine stem cells.(4) The available putative markers are limited and often recognize multiple subpopulations members of a stem cell lineage.(5) There is a lack of reactivity between commercial monoclonal antibodies and epitopes on equine cells therefore phenotyping has been incomplete.(5) Current studies in the equine MSC field have shown positive expression of cell surface markers CD90 and CD44,(6, 7) and negative expression of CD34 and CD45.(8) The presence of surface markers does not however, denote that MSCs are in a completely undifferentiated state.(3) Sorting the MSCs with a tag-less

method would circumvent these CD marker issues that plague the equine MSC researchers.

Mesenchymal stem cells are available for isolation from many different tissues in horses.(9, 10) They are sparse in numbers in post-natal tissues compared to embryonic tissues, creating a need for sorting methods to separate MSCs from differentiated cells in the tissue. Pluripotent MSC subpopulations have been identified in humans(8, 11-13) and rats.(14) These subpopulations have been found to have different shapes, proliferation and differentiation abilities.(14, 15) It is therefore important to be able to isolate the fractions of MSCs that proliferate and differentiate optimally for the application of interest.

Field-flow fractionation (FFF) describes a group of bioanalytical techniques that have applications in the separation of bioanalytes ranging from proteins and nucleic acids to viruses, organelles and whole cells.(3) Gravitational field-flow fractionation (GrFFF) is a type of FFF technique that relies on gravity to achieve sedimentation.(3) Cells differing in molar mass, size and surface antigens are driven by gravity into different velocity regions. The cells are then carried downstream through the channel at different speeds, and exit the channel after different retention times. The distribution of the cells into the various resulting fractions reveals the separation characteristics.(16) After a sedimentation step, equilibrium is reached in traditional GrFFF systems between the gravitational field and the hydrodynamic forces of the transport fluid stream within the capillary channel. Cell sedimentation in the system tends to cause cell adhesion to the wall of the capillary channel and cell–cell aggregation/stacking. Due to the adherent nature of multipotent MSCs, non-equilibrium gravity assisted dynamic fractionation

(NEEGA-DF), which skips the sedimentation step, is utilized to circumvent these MSC tendencies and increasing cell recovery.(2, 17)

GrFFF-based methods have been shown to be potentially useful for cellular applications. The separation of neoplastic B cells from healthy B and T cells in a heterogeneous blood sample has been recently described.(18) GrFFF has also been used to sort different human stem cells(19) and non-equilibrium GrFFF has been implemented to isolate, purify, and sort human MSCs from clinical specimens derived from different sources.(17) Once separated from differentiated cells, the differences in donor tissue MSC source can be distinguished by the different elution profiles. Resulting fractions will have varied commitment potentials which correspond to their differing levels of stem cell-like activity.(3)

Our purpose in this study was to validate the use of non-equilibrium GrFFF as a sorting technique for 1) equine muscle-derived mesenchymal stem cells (MMSCs), 2) periosteal tissue-derived mesenchymal stem cells (PMSCs), 3) bone marrow-derived mesenchymal stem cells (BMSCs), and 4) adipose tissue-derived mesenchymal stem cells (AMSCs).

3.3. Materials and Methods

3.3.1. Samples

Six adult horses (2-5 years of age) were used for post mortem collection of bone marrow, periosteum, skeletal muscle, and adipose tissue. The horses were donated to the Atlantic Veterinary College for reasons other than this study, and were euthanized in accordance with University of Prince Edward Island Animal Care Committee approved

protocols.(Chapter 2) All horses were first sedated with xylazine IV (1.1 mg/kg) (Xylamax, Bimeda, Cambridge, ON) and then euthanized with pentabarbitol sodium injection IV (10 ml/50 kg) (Euthanyl Forte, Bimeda, Cambridge, ON).

3.3.2. Tissue collection and cell isolation

Techniques were performed as described in an earlier study.(Chapter 2) Briefly, immediately after euthanasia, bone marrow (Illinois bone marrow biopsy needle, Carefusion, San Diego, CA) was aseptically collected from the sternebrae, adipose tissue (24 cm^3) from the left subcutaneous supragluteal area lateral to the tail head, muscle (9 cm^3) from the left semitendinosus/membranosus muscles, and periosteum (4 cm^2) from the proximal medial surface of the right tibia. The aspirate (9.5 mL) of bone marrow was collected from the fourth sternebrae into a 12 mL syringe that had been pre-loaded with 2.5 mL of 1000 IU/mL heparin (Leo Pharma Inc., Thornhill, ON). Another sample was immediately drawn from the fifth sternebra in the same fashion and transported to the laboratory. Cells were isolated from bone marrow via a centrifugation gradient technique. The tissues collected were placed in alpha minimal essential media (αMEM, Invitrogen, Toronto, ON) and transported to the laboratory. Cells were isolated from fat, muscle, and periosteum, all by means of same enzyme digestion technique. (Chapter 2)

3.3.3. Cell cryopreservation

Cells were divided into 2.5 million cell aliquots with 1.8 ml of freezing media (10 ml DMSO in 90 ml FBS) in cryo-vials (Corning Incorporated, Corning, NY). They were kept at -80^oC for a minimum of 24 hours and then placed in a liquid nitrogen tank

until removed for cell culture. Viable cells were plated in T-75 (Corning Incorporated, Corning, NY) flasks at a cell density of 33 x 10^3 cells/cm^2 in standard media (SM) (αMEM supplemented with 10% fetal bovine serum (FBS, PAA Laboratories Inc., Etobicoke, ON) , L-glutamine (2 mM)(Invitrogen, Toronto, ON), 10000 U penicillin, 10 mg streptomycin/mL (Invitrogen, Toronto, ON), and 250 μg/mL amphotericin B (Invitrogen, Toronto, ON)).

3.3.4. Cell preparation

Cultured and expanded cells from passage 2 of each of the 4 donor tissue sources (muscle, periosteum, bone marrow, adipose) from 4-6 horses were used for the GrFFF. PMSCs and AMSCs were sorted from 4 horses, while MMSCs and BMSCs were sorted from 6 horses. Cells were washed with phosphate buffered saline (PBS, Invitrogen, Toronto, ON) and then incubated for 30 minutes in a humidified 5% carbon dioxide and 95% air atmosphere incubator at 37°C with 5 parts Versene (Invitrogen, Toronto, ON) to 1 part Trypsin (Invitrogen, Toronto, ON). The reaction was stopped with an equal amount of SM. The cell suspension was spun at 377 x g for 10 minutes and the supernatant removed. The pellet was vortexed and resuspended in 3 ml mobile phase solution (1g BSA, Bovine serum albumin, Fischer Scientific, Fair Lawn, NJ) in 1L PBS made with ultra pure water and 5000 U penicillin, 5 mg streptomycin/mL). Aliquots of 800 x 10^3 cells from each sample were seeded into 6 Eppendorf vials, spun down at 377 x g for 7 minutes, and the supernatant was removed. Fifty microlitres of mobile phase solution was added to each Eppendorf vial and cells were resuspended.

3.3.5. GrFFF System

GrFFF system was purchased from byFlow s.r.l. (byFlow s.r.l, Bologna, Italy) and assembled and operated as per manufacturer's instructions. Sterilization of the fractionation system and the 100-μL HPLC syringe (Hamilton, Reno, NV) to be used for sample loading was performed at the beginning of each working day as previously described and schematized.(2,19) Each aliquot of 800 x 10^3 cells was then individually injected into the GrFFF system(2,17) and sorted into 6 fractions by changing the collection tube every 5 minutes. This timing was based on human MSC sorting work done with the GrFFF system(2) and validation work done in our laboratory that graphed the absorbency readings at different intervals and adjusted the collection times until fraction absorbencies were consistently different from one group to the next. The absorbencies (optical densitiy, OD) of the six fractions were then characterized by spectrophotometric analysis (LKB Biochrom Ultrospec II 4050 UV/Vis Spectrophotometer, Biochrom, Holliston, MA) at a wavelength of 600 nm. The 6 fractions from each of the 6 aliquots were combined to provide pooled fractions that were numerically assessed by hemocytometric anaylsis and seeded at equal concentrations into assays as diagramed in Figure 3.1.

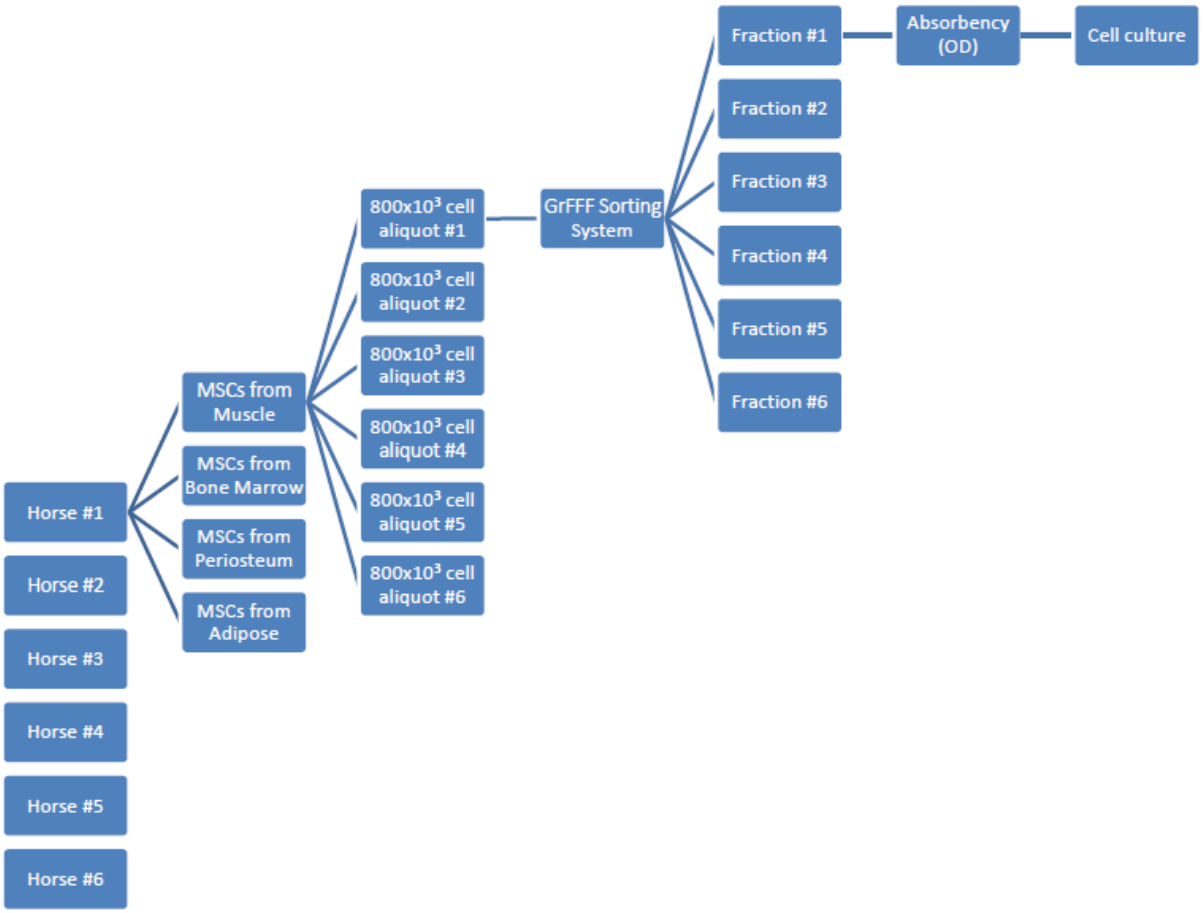

Figure 3.1 Diagram of steps of GrFFF cell sorting study design. Six horses had four tissues collected (bone marrow, adipose, muscle and periosteum) and MSCs cultured from each tissue. Each aliquot of 800 x10^3 cells was then individually sorted by the GrFFF system into 6 fractions. The absorbencies (OD) of each fraction were determined. The sorting process was done on 6 different aliquots and then resulting fractions were combined to provide pooled fractions for further culture.

3.3.6. Characterization of sorted MSCs

Sorted cells from each tissue source were seeded at a density of 1,300 cells/cm^2 into 30 mm tissue culture dishes (Corning Incorporated, Corning, NY) and

supplemented with and maintained for one week in standard medium after which adherence to the flask and spindle shaped morphology was confirmed using direct microscopic analysis at 10x.

3.3.7. Cell differentiation

Cells from each tissue source from each of 3 individuals were induced, as described below, to differentiate into the following three lineages for evaluation: 1.) adipocyte, 2.) chondrocyte, and 3.) osteoblast. Each of the 3 lineages was cultured in parallel with one in standard media as previously described.(Chapter 2) Light microscopy (Axiovert 40 CFL, Carl Zeiss Canada Ltd., Toronto, ON) digital images (Power shot G5, Canon, Mississauga, ON) were taken on day 7 at 10x to assess the different morphologies. Histochemistry and morphology was used to confirm differentiation into the 3 lineages as described below.

3.3.8. Adipogenic differentiation

Cells were seeded at a density of 1,300 cells/cm^2 into 35 mm wells. Cells were first cultured for 3 days in standard medium. Thereafter, the cells were exposed to an adipogenic induction medium (AM) (DMEM/F12 (Invitrogen, Toronto, ON), 3% FBS, 10000 U penicillin and 10 mg streptomycin/ml, 250 μg/ml amphotericin B, 33 μmol/L biotin (Sigma, Oakville, ON), 17 μmol/L pantothenate (Sigma, Oakville, ON), 1 μmol/L insulin (Sigma, Oakville, ON), 1 μmol/L dexamethasone (Sigma, Oakville, ON), 0.5 mmol/L isobutylmethylxanthine (IBMX, Sigma, Oakville, ON), 5 μmol/L rosiglitazone (Toronto Research Chemicals, Toronto, ON) and 5% rabbit serum

(Invitrogen, Toronto, ON)) for 2 days. Thereafter, the same medium without the IBMX and the rosiglitazone was used to maintain the adipocyte cell culture until day 7 when the cells were fixed for 20 minutes in 10% neutral buffered formalin (Fisher Scientific, Nepean, ON) at room temperature and stained for neutral lipid accumulation with Oil Red O indicating adipogenic differentiation. (20)

3.3.9. Chondrogenic differentiation

Cells were seeded at a density of 1,300 cells/cm^2 into 35 mm wells and supplemented with a chondrogenic differentiation medium (CM) (Hams 12 (Sigma, Oakville, ON), dexamethasone (10-7 M), ITS+1(Sigma, Oakville, ON) (culture supplement containing bovine insulin, transferrin, selenous acid, linoleic acid, and BSA) 5% FCS, 10000U penicillin and 10 mg streptomycin/mL, 250 µg/mL amphotericin B, 50 µg/mL ascorbic acid (Sigma, Oakville, ON), 1 ng/mL recombinant human transforming growth factor-beta 1(rhTGF-beta1,Millipore Temecula,CA). Cultures were maintained for 7 days and then fixed for 20 minutes in 10% neutral buffered formalin at room temperature. Cultures were then stained with Alcian blue pH 1.0 for the detection of sulfated proteoglycans to confirm chondrogenic differentiation.

3.3.10. Osteoblastic differentiation

Cells were seeded at a density of 1,300 cells/cm^2 into 35 mm wells. Cells were supplemented with an osteogenic induction medium (OM) (α-MEM, 5% FCS, 10000 U penicillin and 10 mg streptomycin/mL, 250 µg/ml amphotericin B, 50 µg/mL ascorbic acid, dexamethazone 10^{-8}, and 10 mM β-glycerophosphate (Sigma, Oakville, ON)).

Cultures were maintained for 7 days and then fixed for 20 minutes in 10% neutral buffered formalin at room temperature. Cultures were then stained for calcium with von Kossa stain(21) and with the substrate naphthol AS MX-PO4 and Red Violet LB salt for alkaline phosphatase(22) to confirm mineralization and osteoblastic differentiation.

3.3.11. Statistical analysis

Comparison of fraction absorbencies was performed using a paired T-test with horse as the experimental unit. Significance was set at values of $P < 0.05$.

3.4. Results

3.4.1. Spectrophotometric analysis of each fraction

MMSCs, PMSCs, BMSCs, and AMSCs were sorted by GrFFF into six fractions, and this was repeated six times. The fraction contents were compared by spectrophotometric absorbance (Figure 3.2a-d) as an objective comparison of cell number and size. Fractions 1 and 5 OD values were very close to zero (readings in thousandths) due to the cells' small size/number and fraction 6 was zero due to complete lack of cells. Absorbencies of fraction 2 and 3 compared to fractions 1, 4, 5, and 6 for each tissue revealed significant difference ($P<0.5$). In general, fractions with higher absorbencies also had higher cell counts and fractions with lower absorbencies had lower cell counts.

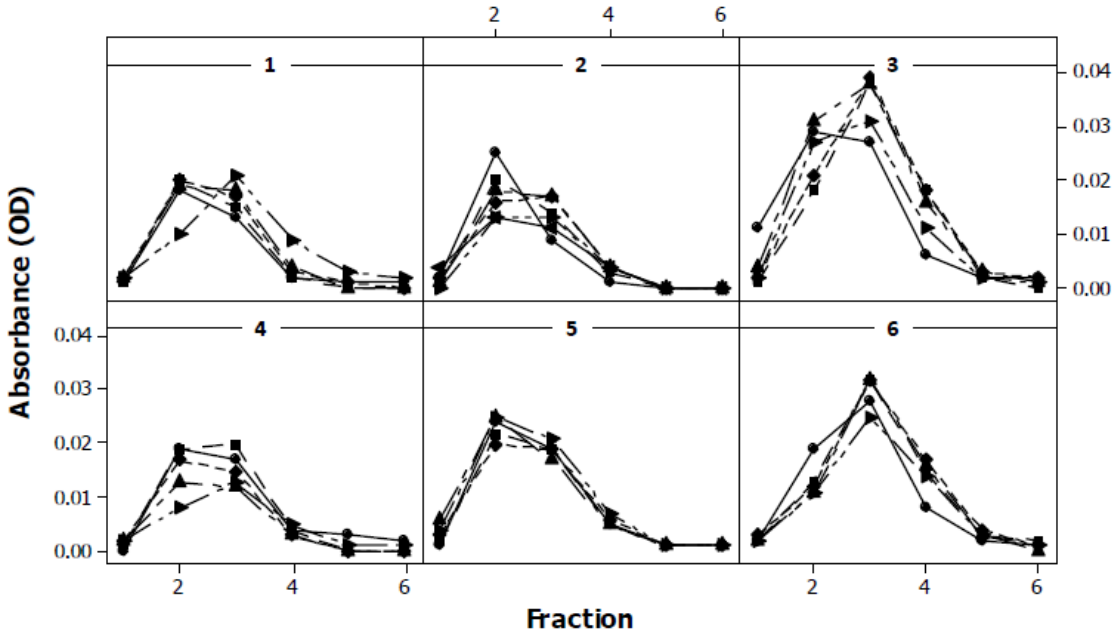

Figure 3.2a Graph of absorbance (OD) vs. fraction for 6 horses (bold numbered panels) and replicates 1-6 (lines) of sorted cells derived from muscle tissue. Note the consistency in absorbency between fraction replicates. Fraction 1 (●), fraction 2 (■), fraction 3 (♦), fraction 4 (▲), fraction 5 (►), fraction 6 (◄).

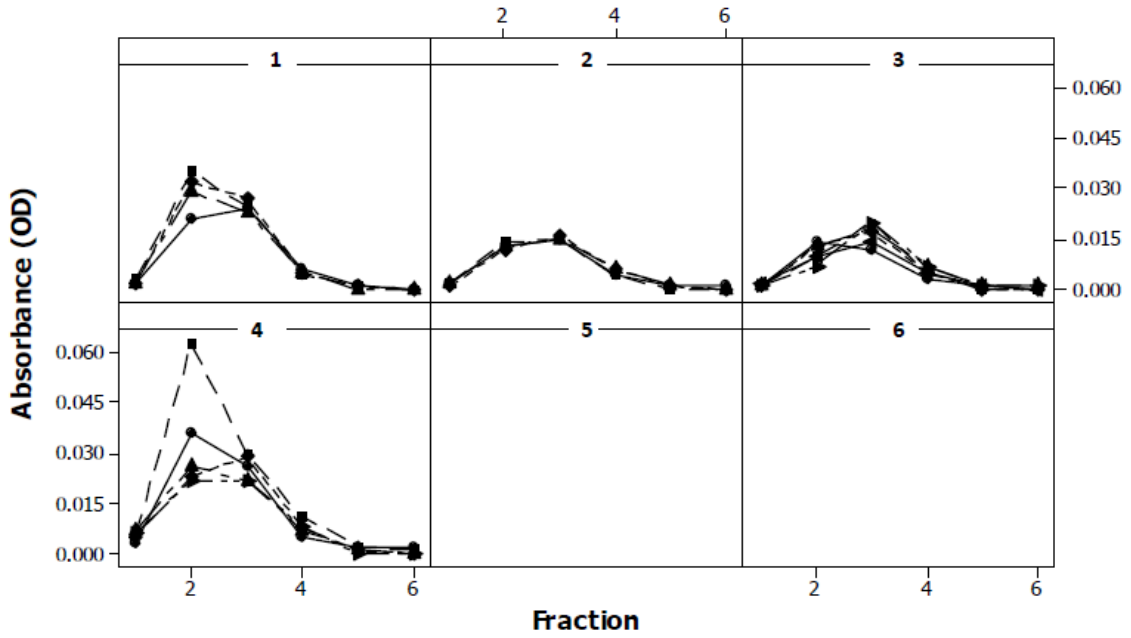

Figure 2b Graph of absorbance (OD) vs. fraction for 4 horses (bold numbered panels) and replicates 1-6 (lines) of sorted cells derived from periosteal tissue. Note the consistency in absorbency between fraction replicates. Fraction 1 (●), fraction 2 (■), fraction 3 (♦), fraction 4 (▲), fraction 5 (►), fraction 6 (◄).

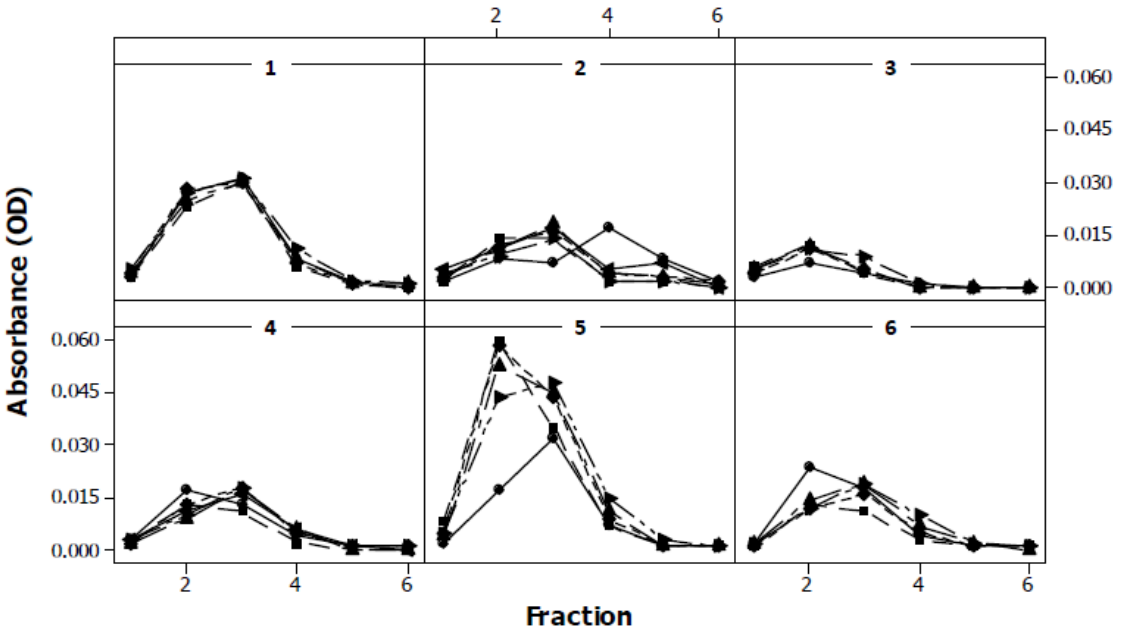

Figure 2c Graph of absorbance (OD) vs. fraction for 6 horses (bold numbered panels) and replicates (1-6) of sorted cells derived from bone marrow. Note the consistency in absorbency between fraction replicates. Fraction 1 (●), fraction 2 (■), fraction 3 (♦), fraction 4 (▲), fraction 5 (►), fraction 6 (◄).

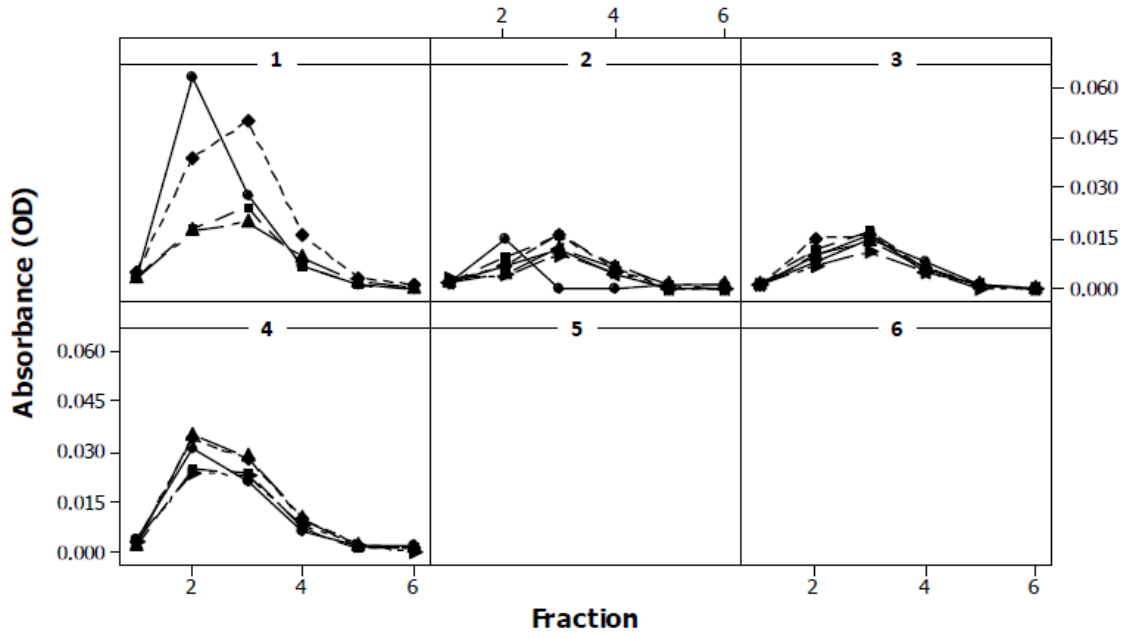

Figure 2d Graph of absorbance (OD) vs. fraction for 4 horses (bold numbered panels) and replicates 1-6 (lines) of sorted cells derived from adipose tissue. Note the consistency in absorbency between fraction replicates. Fraction 1 (●), fraction 2 (■), fraction 3 (♦), fraction 4 (▲), fraction 5 (►), fraction 6 (◄).

3.4.2. Microscopic analysis of each fraction

Each MSC type (MMSCs, PMSCs, BMSCs, and AMSCs) from each horse had cells in fractions 1-5, but none in fraction 6. Cells from all cell sources and fractions adhered to the plastic culture substrate used. The cell recovery from GrFFF sorting system was poor overall and ranged from 28-73%. This range was attributed to inter-horse variation. The highest number of cells was found in fractions 2, 3, and 4 in all tissues. The morphology of cells in fraction 1 was more cuboidal while cells in fractions 2-5 were more classic fibroblastic spindle shapes indicating that different subpopulations

were indeed separated from one another (Figure 3.3). MMSCs, PMSCs, BMSCs, and AMSCs were sorted by non-equilibrium GrFFF while maintaining sterility and viability.

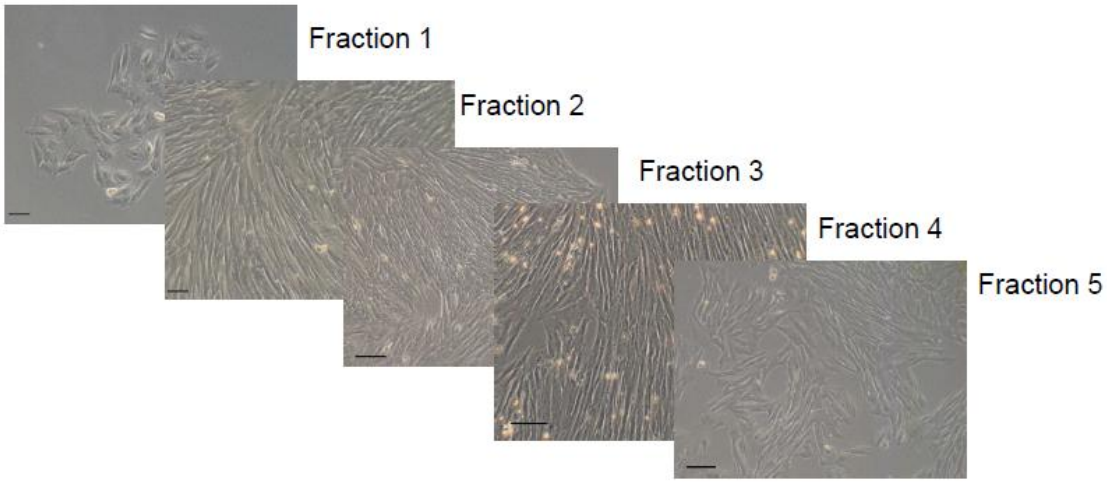

Figure 3.3 Representative photomicrographs of differing morphology between fractions of MSCs cultured from GrFFF sorted equine muscle. Note that the morphology of cells in fraction 1 was more cuboidal in comparison to fractions 2-5 that were more classic fibroblastic spindle shapes. All are unstained. Scale bar 200µm.

3.4.3. Trilineage differentiation

Cells from fractions 1-5 from each tissue were able to undergo tri-lineage differentiation as seen in the representative photomicrographs in Figure 3.4. Cells cultured in adipogenic differentiation medium for 4 days had positive results for oil red O staining of lipid droplets. Cells cultured in standard medium did not develop lipid droplets and lacked staining with oil red O. MSCs cultured in chondrogenic differentiation medium for 7 days stained positively for glycosaminoglycans with Alcian blue. Cells cultured in standard medium lacked Alcian blue stain uptake. Cells cultured

in osteogenic differentiation medium for 7 to 10 days formed bone nodules based on positive results of alkaline phosphatase and calcium specific stains. Cells cultured in standard medium did not develop nodules and lacked staining for alkaline phosphatase and calcium.

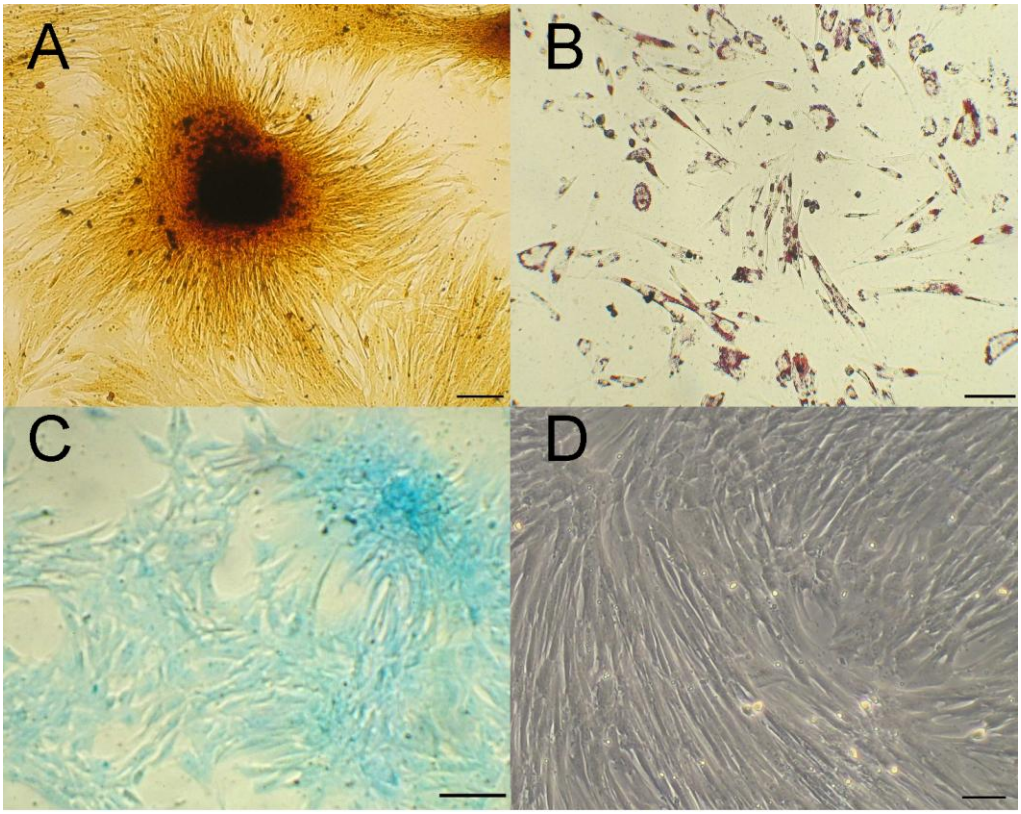

Figure 3.4 Representative photomicrographs of GrFFF sorted MSCs (fractions 1-5 from each tissue) after tri-lineage differentiation and histochemical staining. Osteogenic medium is stained with von Kossa stain (A), adipogenic medium is stained with oil red O (B), chondrogenic medium is stained with Alcian blue (C), and standard medium is unstained (D). Scale bar is 200µm.

3.5. Discussion and Conclusions

This is the first study to validate the use of non-equilibrium GrFFF as a sorting method for equine derived MMSCs, PMSCs, BMSCs, and AMSCs. The cells from each source were driven by gravity into different velocity regions and successfully sorted into fractions revealing that they possess differences in molar mass, size or surface antigens. The fractions with higher absorbencies also had higher cell counts, while those with lower absorbencies had lower cell counts. Cell size likely also plays a part in the absorbency measurements as larger cells travel more quickly through the chamber than do smaller cells.(2) However, in this study we measured cell number and did not measure cell size.

MMSCs, PMSCs, BMSCs, and AMSCs used for non-equilibrium GrFFF sorting in this study were previously characterized by morphology, adherence to polystyrene plastic (tissue culture substrate), tri-lineage differentiation, and stem cell surface marker detection by immunofluorescence and flow cytometric analysis.(Chapter 2) Although populations of cells from each tissue had a high percentage of purity based on the flow cytometry results from this previous work, it became obvious in the present study, that several subpopulations were present in each sample. The populations sorted into fractions that each had different absorbencies, and were therefore concluded to be differing subpopulations. These subpopulations were found to have trilineage differentiation capabilities, thus have not been altered by the sorting process. Evidence from this study indicates that cell sorting by properties other than cell surface markers is essential when the phenotype has not been completely elucidated.

This study aimed at validation of the non-equilibrium GrFFF technique for use with equine MSCs which has not to the author's knowledge been published before.

Other studies using other sorting techniques, other MSC sources, and other species have been reported. One group used equine MCSs derived from intervascular and perivascular umbilical cord matrix and sorted them by a size-sieving method.(23) They were successful in sorting the cells into two homogenous sub-populations and found the large intervascular cells and small perivascular cells to have faster proliferation rates than unsorted controls. The sub-populations however, had similar marker expression and differentiation potential to one another, suggesting that further sorting by cell surface characteristics may have been possible. Another group used the non-equilibrium GrFFF technique with human MSCs to successfully obtain a homogeneous population of MSCs from a heterogenous one, to sort MSCs with dissimilar differentiation potentials, and to distinguish MSCs from different sources that had differing cell surface markers.(17) Roda et. al. showed the cell surface differences definitively using FACS analysis which this research will employ in future investigations of the homogeneous subpopulations that were isolated from a heterogeneous equine MSC population.

Many aspects of the non-equilibrium GrFFF technique make it ideal for use in cell culture. Non-equilibrium GrFFF allows for the improvement of MSC isolation time by clearing other cells and contaminates from the sample in an early passage.(24, 25) Conventionally, this is achieved by adherence and detachment cycles implemented over several passages during cell culture. The shortening of this clean-up phase with a technique such as GrFFF is of great use in clinical application of stem cell therapy as it shortens the return time on samples received for injured patients.(2, 26)

The GrFFF technology is similar to fluorescence activated cell sorting (FACS) in that it is a system to sort subpopulations of cells but it is superior in several aspects. The

non-equilibrium GrFFF is a tag-less system of stem cell sorting that will avoid augmentation of the MSCs.(2) The GrFFF sorting system is also very economical as it can be assembled and maintained in the laboratory from inexpensive instruments and reagents owned by most biotechnology laboratories or purchased for a fraction of the cost of a FACS machine. The GrFFF system is also far less technically difficult to operate than the FACS system. Perhaps most importantly, GrFFF allows for the MSCs to be maintained under sterile conditions also allowing for further culture, expansion, assays, and use in cell based therapies after fractionation.(3) In the current study it was also confirmed that MMSCs, PMSCs, BMSCs, and AMSCs can be sorted by non-equilibrium GrFFF while maintaining viability for further assays.

The main limitation to this system is the low number of cells that can be fractionated per run. Another study discovered a polar hydrophobic environment on the PVC material used in the GrFFF system which explains the low recovery of biological sample. A coating was placed on the polyvinylchloride which improved the sample returns.(27) As we were validating the system for use with equine MSC for the first time, we chose to use the system in its simplest form. We increased sorting throughput by pooling fractions collected at the same retention times from repeated runs. After validation of a single system with human lymphocytes, others have set up two GrFFF channels in parallel to increase sorting throughput.(19)

In equine regenerative medicine, identification of the optimum source of MSCs, or other progenitor cells, for each application has been the focus of much evaluation.(Chapter2) (9, 10, 28, 29) This research points out that the variation of MSCs within each source must also be taken into consideration. Techniques for sorting and

enrichment of MSCs may be the key to isolation of the equine MSC phenotypes. To this end, future goals include using non-equilibrium GrFFF for MSC purification and fractionation into subpopulations to be evaluated for CD markers using flow cytometry. Once the phenotype is elucidated, comparative assays between the fractions will determine the optimum source and CD markers of MSCs for the intended application. With this information the GrFFF system will be employed to isolate the subpopulation of interest allowing for culture and expansion of an ultrapure population.

In summary, the ability to affordably and effectively sort equine MSCs with non-equilibrium GrFFF now broadens the choices available to clinicians using MSCs in cell based therapies.

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4. OSTEOGENIC POTENTIAL OF SORTED EQUINE MESENCHYMAL STEM CELL SUBPOPULATIONS[1]

4.1. Abstract

The objective of this study was to use non-equilibrium gravitational field-flow fractionation (GrFFF), an immunotag-less method of sorting mesenchymal stem cells (MSCs) into subpopulations, to sort equine muscle tissue-derived (MMSC) and bone marrow-derived mesenchymal stem cells (BMSC) into subpopulations and then to perform assays allowing comparison of their osteogenic capabilities. Cells were isolated from left semitendinosus muscle tissue, and from bone marrow aspirates of the fourth and fifth sternebrae from one young, adult horse. Aliquots of 800 $x10^3$ MSCs from each tissue source were sorted into 5 fractions using non-equilibrium GrFFF (GrFFF proprietary system). Equine MMSCs and BMSCs were consistently sorted into 5 fractions that remained viable for use in further osteogenic assays. Pooled fractions were cultured and expanded for use in assays including: flow cytometry; Histochemistry; bone nodule assays; and real time qPCR to identify gene expression of osteocalcin, RUNX2, and osterix. Statistical analysis confirmed strongly significant upregulation of osteocalcin, RUNX2, and osterix for the BMSC fraction 4 with $P<0.00001$. Flow cytometry revealed different cell size and granularity for BMSC fraction 4 and MMSC fraction 2 when compared with unsorted controls (whole population) and other fractions. Histochemisty and bone nodule assays revealed positive staining nodules but no significant differences between tissue sources or fractions. Subpopulations of MSCs exist and have different osteogenic capacities within equine muscle and bone marrow derived sources; therefore, it is important to consider these differences when using equine stem cell therapy to induce bone healing in veterinary medicine.

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4.2. Introduction

Expedited fracture healing in horses is needed due to the inherent risks of contralateral limb laminitis, cyclic loading and fatigue failure of implants,(1) and non-unions due to disturbed blood supply to the injured bone.(2) The most common method to accelerate bone healing in horses is the use of an autogenous, cancellous bone graft.(3) Bone tissue engineering methods, including the use of mesenchymal stem cells (MSCs) to enhance bone healing, have been evaluated in research studies in many species.(4-7) Ectopic bone formation has been verified for MSCs injected subcutaneously in dogs,(8) and porcine maxillary sinus defects have shown increased boney healing when filled with MSCs.(9) The injection of MSCs into non-union fractures of humans has proven to be safe and effective for healing deficits in bone.(10) The injection of MSCs into the distraction gap of rats has been shown to have encouraging results in preventing a non-union.(11) Other *in vitro* studies,(12, 13) have demonstrated the potential for the use of MSC-based treatments in equine musculoskeletal injuries. While MSC therapies show promise, they are in great need of standardization and refinement prior to clinical use to increase their effectiveness and success.

Part of that refinement can be addressed by making efforts to use the purest cultures of stem cells that are optimal at healing the tissue of interest. It is well known that MSCs derived from a single source are made up of a heterogeneous population.(14, 15) Subpopulations of MSCs are documented within many tissue types and species(16)

including mouse bone (17), blood (18) and muscle,(19) rat bone marrow (20) and calvarial bone (21), human heart (22) and adipose (23), equine adipose (24)(Chapter 2), umbilical cord (25), muscle, periosteum, and bone marrow (Chapter 2). These cell subpopulations have been found to have different shapes, proliferation and differentiation abilities. (20, 26) It is therefore important to be able to isolate the fraction of MSCs that proliferates and differentiates optimally for the application of interest.

Identification of the source and subpopulation of MSCs with the best osteogenic potential may prove vital for moving basic science research toward clinical cell-based treatments to promote bone healing.(27) The sorting of cells into their respective subpopulations is traditionally conducted using fluorescence-activated cell sorting (FACS).(28) Due to the high cost, complicated technique, reliance on clearly elucidated cell surface markers,(29) and the cell function altering nature of the FACS process,(30) a more economic and less harsh method is required.

Field-flow fractionation (FFF) encompass a group of bioanalytical techniques that can be used in the separation of bioanalytes ranging from proteins and nucleic acids to viruses, organelles and whole cells.(30) Non-equilibrium gravitational field-flow fractionation (GrFFF) is a flow through type of FFF technique that relies on gravity to sort MSCs(31) based on differences in molar mass, size and surface antigens. The cells settle into different velocity regions of the fluid filled channel, are carried downstream at different speeds, and exit the channel after different retention times. The division of the cells into the various resulting fractions reveals the separation characteristics.(32)

GrFFF-based methods have been shown to be useful for cellular applications. GrFFF has been used to sort human stem cells(33) and non-equilibrium GrFFF has been used to isolate, purify, and sort human MSCs derived from different sources. The distinctions between donor tissue MSC sources may be distinguished by the different elution profiles. Resulting fractions will have an array of commitment potentials that correlate with their differing degrees of stem cell-like potential.(30)

Previous work in our lab has validated the use of a non-equilibrium gravitational field-flow fractionation (GrFFF) MSC sorting system for use with equine MSCs,(Chapter 3) and has shown that muscle and bone marrow may be preferred tissue sources for promotion of bone healing based on osteogenic capacity when compared to periosteum and adipose tissue.(Chapter 2) Based on these findings, we employed the use of the non-equilibrium GrFFF system to sort cells into their respective subpopulations so that the osteogenic capabilities of cells in each subpopulation could be further studied.

We hypothesized that equine muscle and bone marrow derived MSCs would consist of subpopulations that would not have differing osteogenic capabilities. The purpose of the study reported here was to compare the osteogenic capacities of unsorted equine muscle-derived mesenchymal stem cells (MMSCs) and bone marrow-derived mesenchymal stem cells (BMSCs), with subpopulations of MMSCs and BMSCs sorted by a non-equilibrium GrFFF system.

4.3. Materials and Methods

4.3.1. Samples

A two year old Standardbred gelding was used for post mortem collection of bone marrow, and skeletal muscle. The horse was donated to the Atlantic Veterinary College for reasons other than this study, and was euthanized in accordance with University of Prince Edward Island Animal Care Committee approved protocols.(Chapter 2) The horse was first sedated with xylazine IV (1.1 mg/kg) (Xylamax, Bimeda, Cambridge, ON) and then euthanized with pentabarbitol sodium administered IV (10 ml/50 kg) (Euthanyl Forte, Bimeda, Cambridge, ON).

Tissue collection and cell isolation techniques were performed as described in chapter 3. Briefly, immediately after euthanasia, bone marrow was aseptically collected from the sternebrae using a bone marrow biopsy needle (Illinois bone marrow biopsy needle, Carefusion, San Diego, CA) and muscle (9 cm^3) was aseptically collected from the left semitendinosus/membranosus muscle. The aspirate (9.5 mL) of bone marrow was collected from the fourth sternebrae into a 12 mL syringe that had been pre-loaded with 2.5 mL of 1000 IU/mL heparin (Leo Pharma Inc., Thornhill, ON). Another sample was immediately drawn from the fifth sternebra in the same fashion and transported to the laboratory. Cells were isolated from bone marrow via a centrifugation gradient technique.(Chapter 2) The tissues collected were placed in alpha minimal essential media (αMEM, Invitrogen, Toronto, ON) and transported to the laboratory. Cells were isolated from muscle by means of an enzyme digestion technique.(Chapter 2)

4.3.2. Cell cryopreservation

Cells were divided into 2.5 million cell aliquots with 1.8 ml of freezing media (10 ml DMSO in 90 ml fetal bovine serum (FBS)) in cryo-vials (Corning Incorporated,

Corning, NY). They were kept at -80^{o}C for a minimum of 24 hours and then placed in a liquid nitrogen tank until removed for cell culture.(Chapter 2, Chapter 3) (25, 34)Viable cells were plated in T-75 (Corning Incorporated, Corning, NY) flasks at a cell density of 33 x 10^3 cells/cm^2 in standard media (SM) (αMEM supplemented with 10% FBS) (PAA Laboratories Inc., Etobicoke, ON) , L-glutamine (2 mM)(Invitrogen, Toronto, ON), 10000 U penicillin, 10 mg streptomycin/mL (Invitrogen, Toronto, ON), and 250 μg/mL amphotericin B (Invitrogen, Toronto, ON)).

4.3.3. Cell preparation

Cultured and expanded cells from passage 2 of each of the 2 donor tissue sources (muscle, bone marrow) were used for the GrFFF. Cells were washed with phosphate buffered saline (PBS) (Invitrogen, Toronto, ON) and then incubated for 30 minutes in a humidified 5% carbon dioxide and 95% air atmosphere incubator at 37°C with 5 parts Versene (Invitrogen, Toronto, ON) to 1 part trypsin (Invitrogen, Toronto, ON). The reaction was stopped with an equal amount of standard media. The cell suspension was spun at 377 x g for 10 minutes and the supernatant removed. The pellet was vortexed and resuspended in 3 mL mobile phase solution; 1g BSA (Bovine serum albumin, Fischer Scientific, Fair Lawn, NJ) in 1L PBS made with ultra pure water and 5000 U penicillin, 5 mg streptomycin/mL). Six aliquots of 800 x 10^3 cells from each tissue sample source were seeded into individual Eppendorf vials, spun down at 377 x g for 7 minutes, and the supernatant was removed. Fifty microlitres of mobile phase solution was added to each Eppendorf vial and cells were resuspended.

4.3.4. GrFFF system

GrFFF system (byFlow s.r.l, Bologna, Italy) was assembled and operated as per manufacturer's instructions. Sterilization of the fractionation system and the 100 μL HPLC syringe (Hamilton, Reno, NV) to be used for sample loading was performed at the beginning of each working day as previously described.(28, 33) Each aliquot of 800 x 10^3 cells was then individually injected into the GrFFF system(28, 31) and sorted into 5 fractions by changing the collection tube every 5 minutes. This timing was based on human MSC sorting work done with the GrFFF system (28) and validation work done in our laboratory that graphed the absorbency readings at different intervals and adjusted the collection times until fraction absorbencies (optical density), characterized by spectrophotometric analysis (LKB Biochrom Ultrospec II 4050 UV/Vis Spectrophotometer, Biochrom, Holliston, MA) at a wavelength of 600 nm, were consistently different among groups as shown in chapter 3. For each tissue sample source, the 5 fractions sorted from each of the 6 aliquots, and one unsorted group of the whole population, were combined to provide pooled fractions that were assessed by hemocytometric anaylsis for cell count. Pooled fractions were plated in T-75 (Corning Incorporated, Corning, NY) flasks at a cell density of 33 x 10^3 cells/cm^2 in standard media. Each fraction was expanded until there were four T-75 flasks at 80% confluence, about 22.4 million cells, which was enough cells for consistent seeding in osteogenic assays as described below.

4.3.5. Osteoblastic cell differentiation and nodule quantification

Sorted cells and unsorted cells, from each tissue source, were seeded at a density of 1,300 cells/cm^2 into 30 mm dishes (Corning Incorporated, Corning, NY) and induced

to differentiate in parallel with one control in standard media as previously described in chapter 2. Cells were supplemented with an osteogenic induction medium (OM) (α-MEM, 5% FBS, 10000 U penicillin and 10 mg streptomycin/mL, 250 µg/ml amphotericin B, 50 µg/mL ascorbic acid, dexamethazone 10^{-8}M, and 10 mM β-glycerophosphate (Sigma, Oakville, ON)). Cultures were maintained for 7 days and then fixed for 20 minutes in 10% neutral buffered formalin at room temperature.(Chapter 2) Cultures were then stained for calcium with von Kossa stain(35) and with the substrate naphthol AS MX-PO4 and Red Violet LB salt for alkaline phosphatase(36) to confirm mineralization of bone nodules and osteoblastic differentiation. Light microscopy (Axiovert 40 CFL, Carl Zeiss Canada Ltd., Toronto, ON) digital images (Power shot G5, Canon, Mississauga, ON) were taken at 10x on day 7 to count the number of bone nodules formed per 20 randomized fields. Quantification of average area, average perimeter, and average intensity were then measured with computer software (Sigma Scan Pro 5, Systat Software, Inc.,San Jose, CA). Comparisons were made between tissue source, fractions, and unsorted controls.

4.3.6. Flow cytometric analysis of MSC surface markers

Cultured and expanded cells from the second passage of each of the 5 fractions and the one unsorted control from both bone marrow and muscle tissue were used for the flow cytometric analysis. The amount of antibody used in cell labeling section below, was optimized with a cytometer (FACSAria flow cytometer, BD Biosciences, Mississauga, ON).

4.3.6.1. Cell preparation

Cells were washed with PBS solution and then incubated for 15 minutes in a humidified incubator at 5% CO_2 and 95% air at 37°C with a mixture of versene (Invitrogen, Toronto, ON) and trypsin (5:1). (Chapter 2) The reaction was stopped with an equal amount of standard medium. The cell suspension was centrifuged (377 X *g* for 10 minutes), and the pellet then was resuspended and washed in ice-cold 1% BSA in PBS solution. The cell suspension was again centrifuged (377 X *g* for 10 minutes), and the resulting pellet was resuspended in ice-cold 1% BSA in PBS solution, stained with trypan blue to determine viability, and counted for flow cytometric analysis.

4.3.6.2. Cell labeling

One million cells per sample were labeled. For each of the 5 fractions and one unsorted control group, one sample was unstained and served as a negative control sample. Successive samples were labeled with previously validated (29, 37, 38) antibodies (CD45, CD44, CD90, CD34, CD 29, CD14, CD79, and MHCII respectively) that were shown to cross react with equine cells as there are no equine specific antibodies available. The isotypes of these antibodies were used for an internal negative control (Table 4.1). Cells were centrifuged (377 X *g* for 10 minutes), and primary antibodies were added in 1% BSA in PBS solution. Samples were placed on ice and incubated for 45 minutes; samples then were washed in ice-cold 1% BSA in PBS solution and centrifuged (377 X g for 10 minutes). The washing and centrifugation steps were repeated 3 times. Cells were stored at 4°C until flow cytometric analysis. The secondary FITC labeled antibody for CD90 was diluted in 1% BSA in PBS solution and incubated on ice for 30 minutes and then washed in ice-cold 1% BSA in PBS solution

and centrifuged (377 X *g* for 10 minutes). The washing and centrifugation steps were repeated 3 times. Cells were stored at 4°C until flow cytometric analysis (FACSAria flow cytometer, BD Biosciences, Mississauga, ON).

Table 4.1 Characteristics and source of antibodies used for flow cytometric analysis of MSC surface markers and antibody isotypes used for internal negative controls.

CD marker	**Fluorochrome**	**Emission wave-length (nm)**	**Excitation wave-length (nm)**	**Source**
CD34	Allophycocyanin	660	650	AbD Serotec, Raleigh, NC
CD44	Phycoerythrin	667	496	Biolegend, San Diego, CA
CD45	Allophycocyanin	660	650	AbD Serotec, Raleigh, NC
CD90	NA	NA	NA	Accurate Chemical & Scientific Corporation, Westbury, NY
CD14	R Phycoerythrin-cyanine 5.1	693	565	Beckman Coulter Canada, LP, Mississauga, ON
CD29	R Phycoerythrin-cyanine 5.1	693	565	Biolegend, San Diego, CA
MHCII	FITC	519	495	AbD Serotec, Raleigh, NC
CD79	Alexa Fluor 647	665	650	AbD Serotec, Raleigh, NC
IgG2a (mouse)	R Phycoerythrin-cyanine 5.1	693	565	Beckman Coulter Canada, LP, Mississauga, ON
IgG1k (mouse)	Phycoerythrin	667	496	Biolegend, San Diego, CA
IgG1 (mouse)	Allophycocyanin	660	650	AbD Serotec, Raleigh, NC
IgG2bk (rat)	Phycoerythrin	667	496	Biolegend, San Diego, CA
IgG2b	Phycoerythrin	667	496	AbD Serotec, Raleigh, NC

IgG1 (mouse)	Alexa Fluor 647	665	650	AbD Serotec, Raleigh, NC
IgM (mouse)	FITC	519	495	AbD Serotec, Raleigh, NC
IgG1 (mouse)	FITC	519	495	AbD Serotec, Raleigh, NC

NA = Not applicable.

4.3.7. Real time quantitative PCR (qPCR)

Cultured and expanded cells from the second passage of each of the 5 fractions and the one unsorted group, from both bone marrow and muscle tissue, were seeded in 6-well plates at 200 cells/cm^2 in triplicate. Half of the wells were induced with osteogenic medium, and the other half were maintained in standard medium to serve as control cultures. Growth of the paired cultures was stopped on day 7 and total RNA was extracted (Aurum total RNA mini kit, Bio-Rad Laboratories, Hercules, CA) from the cells. The cDNA was synthesized from total RNA via a cDNA synthesis kit (iScript cDNA synthesis kit, Bio-Rad Laboratories, Hercules, CA) Primers derived from the coding regions of osteocalcin were as follows: forward, 5'-CTGGGCCAGGACTCCGCATCT-3'; and reverse, 5'-AGCCAGCTCGTCACAGTCTGGGTTG-3'. Primers derived from the coding regions of RUNX2 were as follows: forward, 5'-CAGACCAGCAGCACTCCATA-3'; and reverse, 5'-CAGCGTCAACACCATCATTC-3'. Primers derived from the coding regions of osterix were as follows: forward, 5'-GGCTATGCCAATGACTACCC-3'; and reverse, 5'-GGTGAGATGCCTGCATGGA-3'. Expression of the osteocalcin (OCN), RUNX2, and osterix genes were quantified via real-time PCR assay with a mix (iQ SYBR Green Supermix, Bio-Rad Laboratories, Hercules, CA) The PCR assay was

performed on a thermal cycler (Rotorgene-6 RG 3000, Corbett Research, Montreal, QC). Cycling conditions were as follows: 95°C for 5 minutes; 35 cycles of 95°C for 15 seconds, 56°C for 30 seconds for OCN (57.7^{o}C for 30 seconds for RUNX2 and osterix), 72°C for 45 seconds, and melting from 55° to 99°C. Nuclease-free water instead of cDNA was used as a negative control sample. The housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used to normalize the expression of each gene of interest.(39, 40)

4.3.7.1. qPCR statistical analysis

The data comprised gene expressions across 5 fractions and 1 unsorted control obtained from 2 tissues of 1 horse, with 3 replicate samples taken from each tissue. The study design had a split-plot character with samples within tissues representing whole-plots and fractions within each sample corresponding to sub-plots. Gene expression was quantified with real-time quantitative PCR. The outcome was computed using the comparative C_T method,(41, 42) or 2 to the power of the negative difference of housekeeping gene (GAPDH) and gene of interest (GOI) CT values to a baseline medium (SM). Outcomes for 3 genes (OCN, Runx, osterix) were analysed separately. The analysis used linear mixed models with random effects of samples (within tissues), after cubic root transformation of the outcome. The linear mixed model assumptions were validated by residual analysis and found to be acceptable for all genes with this particular transformation. The linear mixed model included fixed effects of tissues and fractions as well as their interaction. Least squares means were back-transformed to the scale of the outcome as estimated medians with 95% confidence intervals. The

statistical analysis was carried out using SAS (proc mixed) software (SAS, version 9.2, SAS Institute Inc, Cary, NC), and the significance level was set at $P<0.05$.

4.4. Results

4.4.1. Microscopic analysis of each fraction

Both MMSCs and BMSCs had cells in each of the sorted fractions 1-5. Cells from all cell sources and fractions adhered to polystyrene plastic. The cell recovery from GrFFF sorting system was approximately 50%. The highest number of cells was found in fractions 2, 3, and 4 from both tissues.

4.4.2. Nodule quantification

BMSC fractions 2, 4, and 5 had noticeably more von Kossa and alkaline phosphatase positive nodules present and BMSC fractions 1, 3, and the unsorted control BMSC population had fewer. Each of the 5 MMSC fraction cultures had noticeably more von Kossa and alkaline phosphatase positive nodules present and the unsorted control MMSC population had fewer. Figure 4.1 shows representative photomicrograph of von Kossa and alkaline phosphatase positive nodules. No substantial differences in average nodule area, perimeter, or stain intensity were noted between tissues or fractions.(Table 4.2) Average intensity is a measure of amount of light that reaches the camera. Therefore, higher intensities represent less stain uptake of nodules and lower intensities represent more stain uptake of nodules.

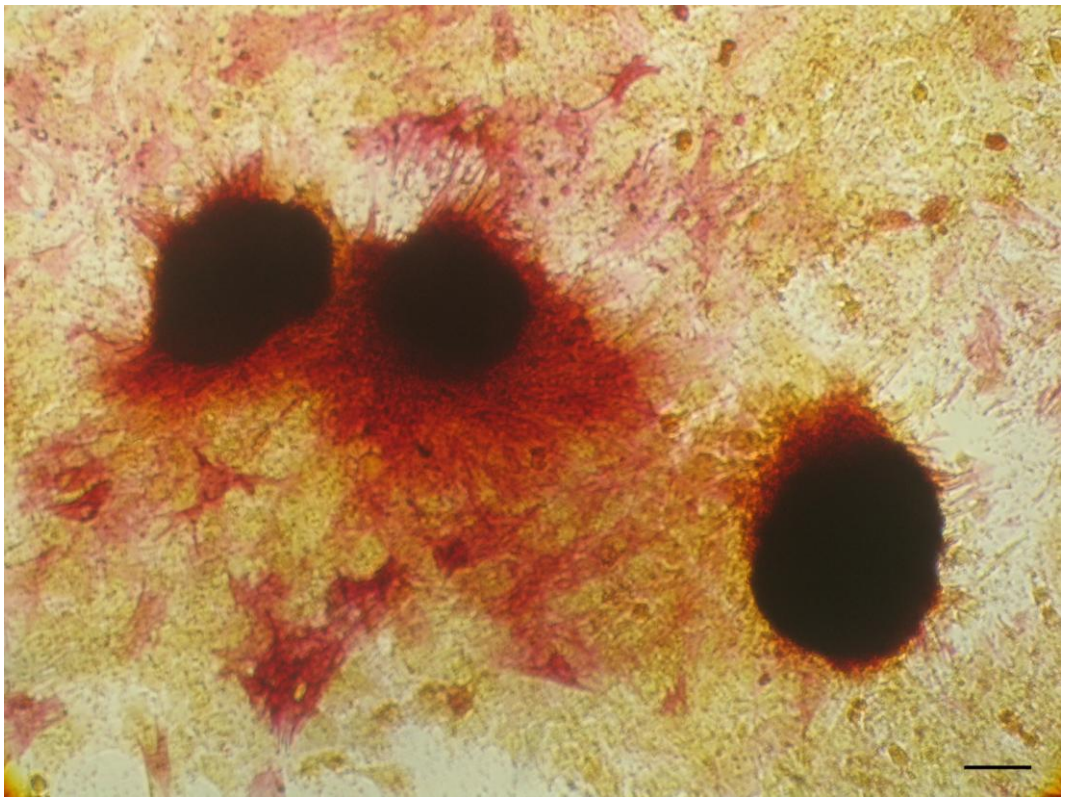


Figure 4.1 Representative photomicrograph of von Kossa and alkaline phosphatase positive histochemical staining for equine muscle derived mesenchymal stem cells cultured from fraction 1. Magnification 10X. Bar = 200 µm.

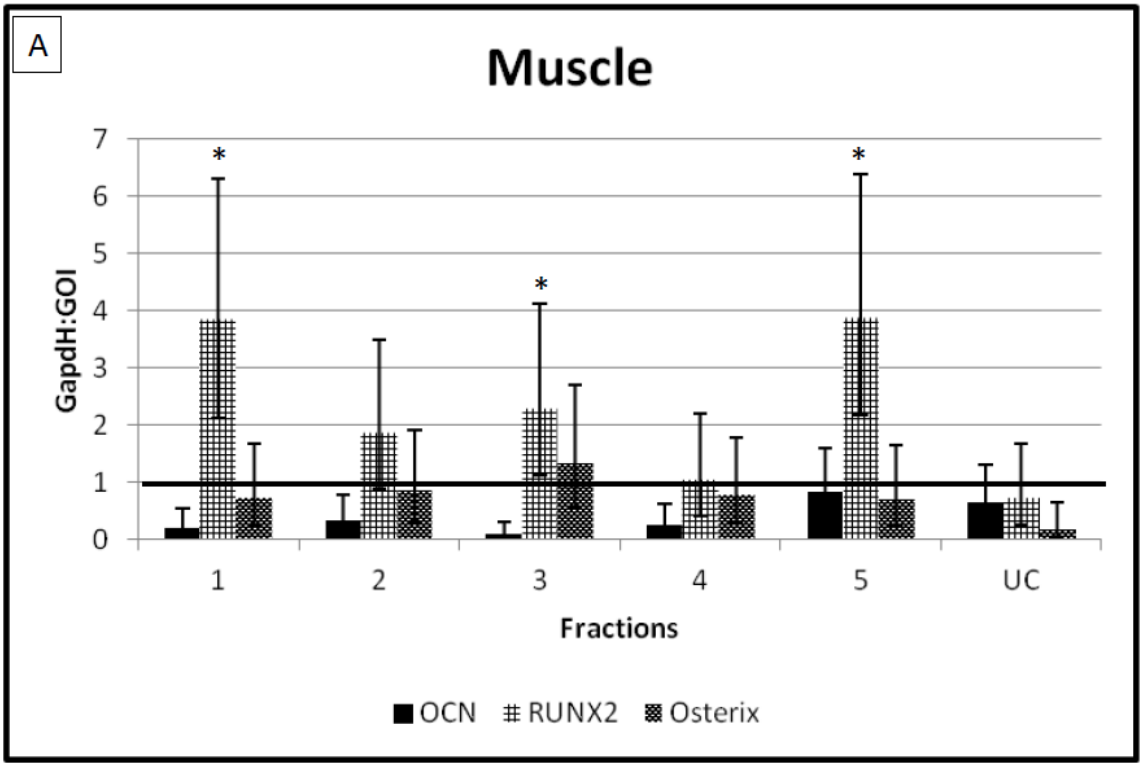
Table 4.2 Raw data of nodule quantification. UC= Unsorted control cells. SD= Standard deviation.

Tissue	Fraction	Number of nodules in 20 fields	Mean area (mm^2) ±SD	Mean intensity ±SD	Mean perimeter (mm) ±SD
Muscle	1	23	0.1 ± 0.7	40 ± 9	1.2 ± 0.4
Muscle	2	8	0.16 ± 0.1	37 ± 7	1.5 ± 0.6
Muscle	3	4	0.07 ± 0.03	43 ± 4	1 ± 0.2
Muscle	4	2	0.23 ± 0.004	38 ± 5	1.9 ± 0.02
Muscle	5	12	0.03 ± 0.01	42 ± 5	0.6 ± 0.2
Muscle	UC	0	0	0	0
Bone Marrow	1	0	0	0	0
Bone Marrow	2	18	0.06 ± 0.02	40 ± 6	1 ± 0.2
Bone Marrow	3	0	0	0	0
Bone Marrow	4	7	0.03 ± .03	42 ± 4	0.7 ± 0.3
Bone Marrow	5	8	0.09 ± 0.03	31 ± 8	1.2 ± 0.3
Bone Marrow	UC	5	0.09 ± 0.05	26 ± 4	1.2 ± 0.3

4.4.3. Real time quantitative PCR

Osteogenic capacity was determined on the basis of gene expression of OCN, RUNX2, and osterix measured in sorted fractions 1-5, as well as in unsorted control, from both tissues (muscle and bone marrow). MMSC fractions 1, 3, and 5 had significantly higher ($P<0.05$) RUNX2 expression after differentiation with osteogenic medium than did the control samples cultured in standard medium. There were no significant differences in OCN or osterix expression between differentiated and nondifferentiated cultures of MMSCs. The BMSC fraction 3 had significantly higher ($P<0.05$) OCN, RUNX2, and osterix expression after differentiation with osteogenic

medium than did the control samples cultured in standard medium. BMSC fraction 5 had significantly higher ($P<0.05$) OCN, and RUNX2 expression after differentiation with osteogenic medium than did the control samples cultured in standard medium. BMSC fraction 4 had highly significant ($P\leq0.00001$) OCN, RUNX2 and osterix expression after differentiation with osteogenic medium in comparison with the control samples cultured in standard medium. The OCN expression was 20 fold that of cells in SM. The RUNX2 and Osterix expression was 57 fold that of cells in SM. None of the unsorted control MMSCs or BMSCs showed significant differences in OCN, RUNX, or osterix expression between differentiated and nondifferentiated cultures of MMSCs. (Figure 4.2A-B)



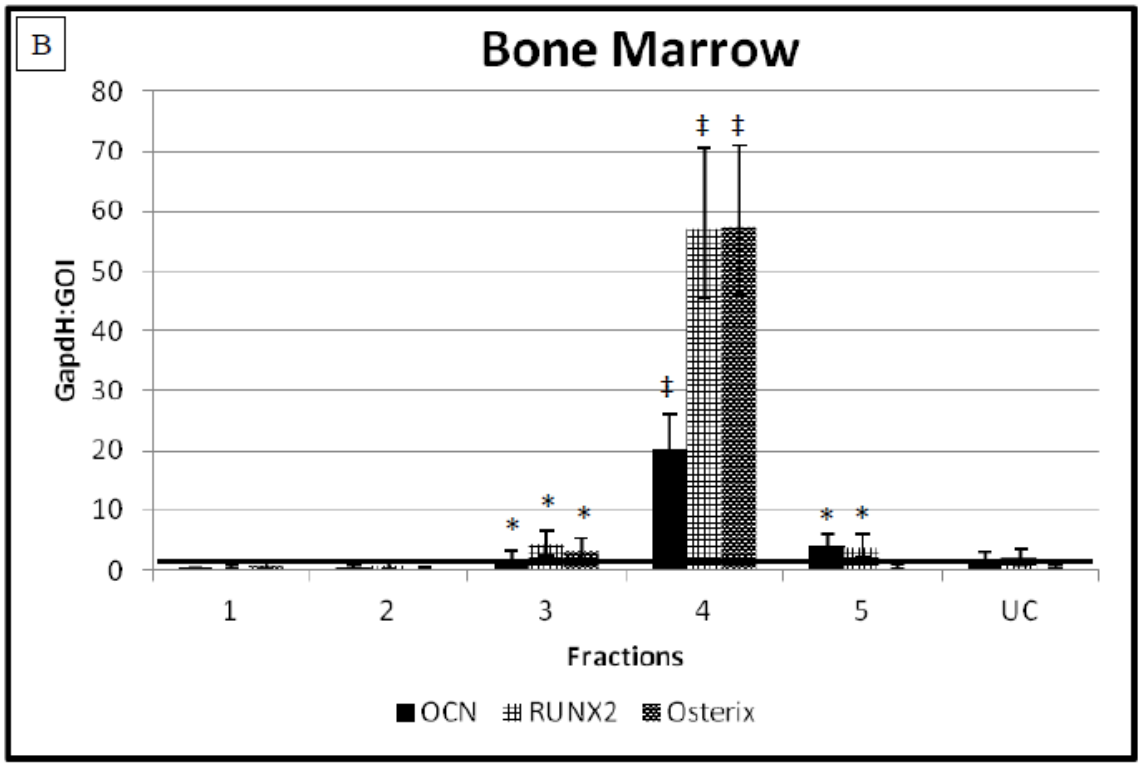


Figure 4.2 Graphical representation of qPCR data. Back-transformed estimates with 95% confidence intervals from statistical model for real-time PCR assay of OCN, RUNX2, and osterix gene expression for 5 sorted fractions (F1-5) of MSCs and one unsorted control (UC) derived from muscle (A) and bone marrow (B). Note the extremely high expression of all three osteogenic markers in bone marrow fraction 4 when compared to other tissue and fractions. Note the difference in x-axis scales between muscle and bone marrow graphs. * Significant ($P \leq 0.05$) expression of gene of interest compared to cultures in standard medium (horizontal black line). ‡Highly significant ($P \leq 0.00001$) expression of gene of interest compared to cultures in standard medium. GapdH: GOI = Ratio of glyceraldehyde 3-phosphate dehydrogenase gene to the gene of interest.

4.4.4. Flow cytometric analysis

Cells isolated from the second passage of each of the 5 fractions and the one unsorted control from both bone marrow and muscle tissue were cultured via standard conditions and each positively expressed CD90, CD44 and CD29 and lacked expression of CD45, CD34, CD14, CD79, and MHCII as determined on the basis of flow cytometric data. Triple stained combinations were highly positive for CD29-CD44, CD29-CD90, and CD44-CD90 expression. Triple stained combinations were negative for CD14-CD79, CD14-MHCII, and CD79-MHCII expression. Results were similar across tissues, fractions and unsorted controls. The unstained flow cytometric analysis for assessment of the granularity and size of cells in the fraction subpopulations, and unsorted whole population, revealed a distinctly different population in bone marrow fraction 4 and muscle fraction 2 (Figure 4.3).

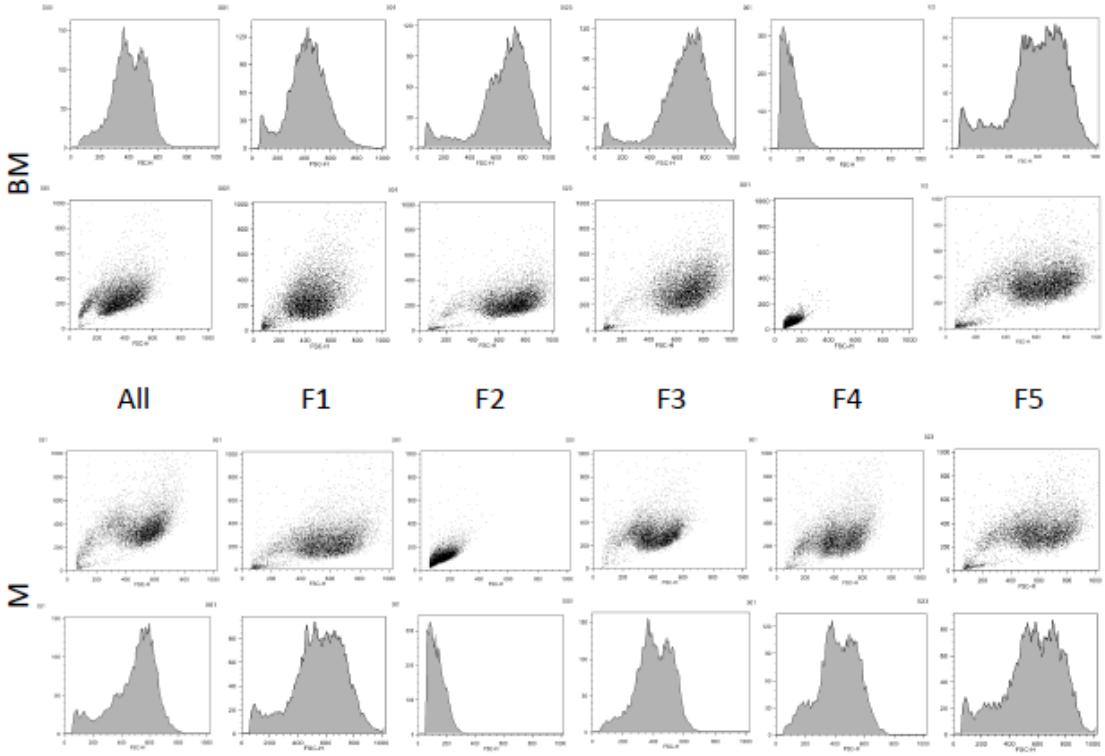


Figure 4.3 Results of flow cytometric analysis of MSCs cultured from five sorted fractions (F1-5) and one unsorted control (UC) derived from equine muscle (M), bone marrow (BM) and unstained for assessment of the population granularity (x axis) and size (y axis). Dot plot and corresponding histogram graphical representation of the subpopulations depict a distinctly different population in bone marrow fraction 4 and muscle fraction 2. The fluorescence intensity (arbitrary units) is depicted.

4.5. Discussion and Conclusions

This is the first study to compare the osteogenic capabilities of subpopulations of equine derived MMSCs and BMSCs sorted using non-equilibrium GrFFF or any other sorting system. As expected, several subpopulations with varying degrees of osteogenic potential were present in each tissue. The fractions of cells had different osteogenic

gene expression based on qPCR, and different cell size and granularity based on flow cytometry. These subpopulations were previously found to have trilineage differentiation capabilities, thus have not been altered by the sorting process.(Chapter 3) Across all tissues, fractions and unsorted controls, all cells' surface markers were consistent with what one would expect for MSCs.(29, 38) Therefore, sorting systems that rely on the cell surface markers alone may have missed the subpopulation differences found in this study. Evidence from this study indicates that cell sorting by properties other than cell surface markers is essential when the phenotype has not been completely elucidated.

Interestingly, most of the fractionated BMSC and MMSC cultures had more von Kossa and alkaline phosphatase positive nodules present than the unsorted control BMSC and MMSC populations. This revealed how very differently the same population of cells acts when separated into subpopulations. In this case the subpopulations displayed more osteogenic activity than did the population as a whole. We believe this finding is explained by the concept of a MSC microenvironment(43-46) that is a niche made up of other cells, soluble molecules, and other naïve MSCs.(46) When this niche is altered by sorting cell populations into subpopulations as was done in this study, it is reasonable to expect the subpopulations to behave differently from one another and from the original unsorted population.(46, 47)

RUNX2 is upregulated alone in the MMSCs subpopulations. Perhaps these cells are at an earlier stage of differentiation than the BMSCs and therefore only express RUNX2. In comparison, particularly in fraction 4, all three genes are upregulated in the BMSCs. Perhaps these BMSCs are more mature and therefore have a more developed

gene expression profile. Also the degree of upregulation of fraction 4 of the BMSCs is impressive with the OCN expression at 20 fold that of the SM control, and RUN2 and Osterix expression at 57 fold that of the SM control. Potentially this would be the ideal population to target in future studies.

The lack of difference between fractions, tissues, and unsorted controls in nodule assays paired with the very significant differences in gene expression can potentially be attributed to the relative disparity in sensitivity between qPCR assays and nodule assays. As the nodule assay is not as sensitive as the qPCR assay, it is possible that more nodule data points would be required before revealing any significant differences. Also nodule quantification is done in 2 dimensions while the nodule itself it a 3 dimensional entity. There could be a loss of information due to the fact that the height of the nodule was not measured.

The main limitation of this study was the use of only one horse. Inter-horse variation can be significant and could affect the results. The findings need to be evaluated in more horses in the future. The main drawback of using this sorting system is the low number of cells that can be fractionated per run.(28) This study increased sorting throughput by pooling fractions collected at the same retention times from repeated runs as described in chapter 3. An expansion step after the sorting process was also employed, and was a simple way of compensating for the low initial throughput. Others have set up two GrFFF channels in parallel to increase sorting throughput,(33) and placed a coating on the PVC in the GrFFF system that improved the sample returns.(48)

In equine regenerative medicine, identification of the source of MSCs with the optimum osteogenic capability has been the focus of much evaluation.(Chapter 2) (27, 49-51)It is evident that the variation of MSCs within each source must also be taken into consideration.(Chapter 3) (25)Techniques for sorting and enrichment of MSCs appear vital to the isolation of the equine MSC phenotypes that are ideal for bone healing.

In summary, subpopulations sorted from equine MMSCs and BMSCs with GrFFF differ in their osteogenic potential. MMSCs may be more appropriate for stimulation of regeneration of other tissues types. Whereas, bone marrow derived MSCs from fraction 4 using the method described here, may have greater bone healing potential because they express high level of osteogenic gene markers. Further clarification of MSC subpopulation phenotypes is necessary to determine the optimum ultrapure population for the intended application.

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5. CONCLUSION

5.1 Characterization and proliferation

This study confirmed that cells derived from equine muscle and periosteal tissues can be characterized as MSCs as determined by morphology, adherence to polystyrene plastic (cell culture substrate), trilineage differentiation, and the detection of stem cell surface markers with immunofluorescence and flow cytometric analyses. It was found that equine MMSCs and PMSCs have superior proliferative capacity to that of BMSCs. Analysis of data from the present study revealed that BMSCs proliferated more slowly than did MMSCs, PMSCs, and AMSCs. This is consistent with previous findings that muscle-derived cells yield greater cell culture numbers within a shorter time frame than do bone marrow–derived cells.(1, 2) In light of this, bone marrow may not be the optimum tissue for use in bone healing when time to culture clinically useful numbers of autogenous cells is considered. The slow proliferation of bone marrow–derived MSCs could have been attributable to the low number of proliferative cells in bone marrow aspirates. On the basis of a CFU fibroblast assay, the frequency of MSCs in the mononuclear cell fraction of equine marrow is reported to be 1 in 4.2 X 10^3 cells and to differ among horses by 10-fold.(3) The small fraction of proliferative cells among the total cell isolates may contribute to the 1 to 2 weeks of extra expansion time needed for bone marrow–derived MSCs over the expansion time required for other equine sources of MSCs.(4, 5) This points to the need for a method to isolate and concentrate this small proliferative fraction of cells before culture. This has been addressed in one equine study using a bone marrow processing system designed for humans, and the results were

encouraging with a reported red blood cell reduction of 92±3%, and marrow mononuclear cell recovery of 91±15%.(6) The limitations were the high cost of the system required to achieve this reduction, the large amount of bone marrow needed for the system to function, and the fact that the system was not fully optimized for equine samples. This study left room for improvement in the cell-sorting method used for removal of contaminant cells that physically impede MSC adherence and thereby hasten the expansion process for bone marrow and other tissue sources.(7)

5.2 Stem cell sorting systems

To this end, this study validated the use of non-equilibrium GrFFF as a sorting method for equine derived BMSCs as well as MMSCs, PMSCs, and AMSCs. The cells from each source were driven by gravity into different velocity regions, then fractions, based on differences in mass, size, and surface antigens. Other sorting systems using counterflow centrifugal elutriation have been applied to human umbilical cord-derived stem cells to obtain homogenous subpopulations based on size, morphology, and proliferative activity.(8) A method based on cell size has been validated with equine MSCs and confirmed the ability to isolate more rapidly proliferating MSC subpopulations from equine umbilical cord matrix.(9) Currently fluorescence-activated cell sorting (FACS) is the standard for sorting populations and subpopulations of cells.(7) However, cells are tagged by antibodies and fluorescent labels in preparation for FACS thus changing their cell surface characteristics and impeding post-FACS assays.(10) Therefore the GrFFF technology is superior because it is a tag-less system of stem cell sorting that will avoid augmentation of the MSCs.(7) Perhaps most

importantly, GrFFF allows for the MSCs to be maintained under sterile conditions also allowing for further culture, expansion, assays, and use in cell based therapies after fractionation.(10) In this study I confirmed that equine derived MMSCs, PMSCs, BMSCs, and AMSCs can be sorted by non-equilibrium GrFFF while maintaining viability for further assays. Future research could be aimed at comparing sorting techniques using equine MSCs but with the current state of the literature, the sorting techniques are difficult to compare because they sort based upon different parameters.

5.3 Optimum source for osteogenic capactity

In equine regenerative medicine, identification of the source of MSCs with the optimum osteogenic capability has been the focus of much evaluation.(11-14) It is unlikely that a single donor source of MSCs will be superior for regeneration of tissue from all different germ layers.(15) This study showed MMSCs and PMSCs have osteogenic potential comparable to that of AMSCs and BMSCs, therefore broadening the MSC source options for clinicians. However, it is well known that MSCs derived from a single source are made up of a heterogeneous population and therefore evident that the variation of MSC phenotypes within each source must also be taken into consideration.(9)

Across all tissues, fractions and unsorted controls, all cells' surface markers were consistent with what one would expect for equine MSCs based upon the current published works in the field.(16, 17) Although there are many CD markers that have been evaluated, the findings of the work described in this thesis and by others show equine MSCs cells are most consistently positive for CD 90, CD 44, and CD 29 and

negative for CD 34, CD 45.(16-18) In the human MSCs field, the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy have defined the minimal criteria for marker expression.(19) In the equine MSCs field, definition of minimal criteria has not been reached due to lack of antibody cross reactivity, specific equine monoclonal antibodies, and therefore a lack of proper immunophenotyping.(17, 20) No specific immunophenotype was elucidated in the findings of this thesis, however it was evident that the subpopulations were different in size and granularity based on flow cytometry of the unstained subpopulations. Interestingly, the subpopulations differed in osteogenic capability as well. Gene expression of osteocalcin (OCN), Runx2, and osterix were quantified with qPCR revealing several fractions from both MMSCs and BMSCs to be significantly increased, some highly, from the baseline expression in standard media. However, the nodule quantification findings did not reflect the outstanding difference that was noted in the gene expression profiles. Perhaps this can be attributed to the fact that the nodule quantification assay is far less sensitive than qPCR and might therefore have only shown significance with a higher n. The subpopulation differences established in this study are important findings that sorting systems relying on the cell surface markers alone may have missed. One group attempted flow cytometric characterization of equine AMSCs in order to elucidate the phenotype of these MSCs, only to conclude that "further insights are necessary to optimize cell phenotype definition, to identify specific subpopulations and to make results among laboratories completely reproducible."(21) Another group demonstrated convincingly that several cell surface molecule changes took place during the first few weeks of establishment of MSC populations, which may

account for the elusiveness of a phenotype amongst researchers.(22) Evidence from the work presented in this thesis indicates that when the exact phenotype of interest is unknown, cell sorting by cell surface markers could be combined with sorting by other properties, as done with GrFFF, to attempt elucidation of that phenotype.

5.4 Microenvironment or niche

It is expected that a fraction of a population should have similar properties to the whole population. However interestingly, most of the fractionated BMSC and MMSC cultures had von Kossa and alkaline phosphatase positive nodules present and the unsorted control BMSC and MMSC populations had fewer nodules present. Also, BMSC fraction 4 had highly significant ($P \leq 0.00001$) OCN, RUNX2 and osterix expression after differentiation with osteogenic medium in comparison with the control samples cultured in standard medium while none of the unsorted control MMSCs or BMSCs showed significant differences in OCN, RUNX, or osterix expression. These findings revealed how very differently the same population of cells acts when separated into subpopulations. In this case the subpopulations displayed more osteogenic activity than did the population as a whole. This finding is consistent with another group who found that some fractions of non-equilibrium GrFFF sorted human adipose derived MSCs had more adipogenic activity than the unsorted control MSCs.(23) I hypothesize that this finding may be explained by the concept of a MSC microenvironment(24-27) that is a niche made up of other cells, soluble molecules, and other naïve MSCs.(27, 28) When this niche is altered by sorting cell populations into subpopulations, as was done in this study, it is reasonable to expect the subpopulations to behave differently from one

another and from the original unsorted population. Obviously there may be great value in this *in vitro* finding, but interpretation must be done with care as the *in vitro* environment is not always representative of the *in vivo* environment that is in fact the long term milieu for these cultured cells.(29)

5.5 Limitations

One of the limitations of the present study was the use of a single osteoblastic marker in the characterization phase of the experiments. Evaluation of the expression of additional genes as osteoblastic markers may have highlighted differences in osteogenic potential among tissues, considering that no significant differences were detected with use of one marker. However, as the primary focus of this study was to confirm osteogenic differentiation as part of trilineage differentiation, osteocalcin was used as the sole marker.(30, 31) Finally, although there was no significant difference ($P = 0.17$) in osteocalcin expression among the 4 unsorted tissues of the 10 horses, unsorted MMSCs showed the highest osteocalcin expression, and it is possible that an increased number of horses may yield data in which the differences were significant. Subsequent experiments added two other markers and the varied findings were indication of the importance of using more than one marker. The main limitation to the sorting phase of the experiments was the low cell throughput of the GrFFF sorting system. To compensate for the low throughput, we pooled fractions collected at the same retention times from repeated runs, and added an expansion step after the sorting process. We found these techniques resolved the issue easily. Finally, the use of only one horse in the last phase of the project assigns a low power to the analysis due to the possibility of

inter-horse variation, but does provide some preliminary evidence validating this approach.

5.6 Future goals

While the MMSCs were shown to have an ideal proliferative capacity, fraction 4 of the BMSCs have a very desirable gene expression profile. This begs the question as to whether future emphasis should be placed on upscaling production of this pro-osteogenic subpopulation. Also, it is necessary to use more horses to repeat the portion of the experiment using non-equilibrium GrFFF for MSC purification and fractionation into subpopulations for further analysis of CD markers using flow cytometry. More markers should be employed using a DNA microarray in efforts to elucidate the phenotype of the optimum subpopulation. With this information the GrFFF system would be employed to isolate the subpopulation of interest allowing for culture and expansion of an ultrapure population. An *in vivo* osteogenic assessment would be called for first in a rodent model and then in a large animal model. Potentially our results would be helpful for future research to use in sample size calculation to determine how many more horses the study should be repeated in to reach an appropriate power, evaluate inter-horse variation, and standardize a protocol for use of the non-equilibrium GrFFF system clinically.

5.7 Summary

To our knowledge, the study reported here is the first to confirm osteocalcin expression in equine muscle- and periosteum-derived MSCs, which indicates their

osteogenic potential. While the characterization of muscle- and periosteum-derived MSCs broadens the choices available to clinicians who use MSCs in cell-based treatments, the heterogenous nature of MSC populations must remain a central focus in investigations of this nature. As shown here, subpopulations sorted from equine MMSCs and BMSCs with GrFFF, differ when it comes to osteogenic potential. Therefore, techniques for sorting MSCs appear vital to isolate, and phenotype, the optimum homogeneous subpopulation that is ideal for equine bone healing. The ability to affordably and effectively sort equine MSCs with non-equilibrium GrFFF, as shown in this study, is promising for its future application in cell-based treatment of bone fractures in horses.

5.8 References

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